

AD _____

Award Number: DAMD17-02-1-0712

TITLE: Skin Bioengineering: Noninvasive Transdermal Monitoring

PRINCIPAL INVESTIGATOR: Richard H. Guy, Ph.D.

CONTRACTING ORGANIZATION: University of Geneva
CH-1211 Geneva 4, Switzerland

REPORT DATE: January 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050603 123

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE January 2005	3. REPORT TYPE AND DATES COVERED Final (1 Jan 2003 - 31 Dec 2004)	
4. TITLE AND SUBTITLE Skin Bioengineering: Noninvasive Transdermal Monitoring			5. FUNDING NUMBERS DAMD17-02-1-0712	
6. AUTHOR(S) Richard H. Guy, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Geneva CH-1211 Geneva 4, Switzerland E-Mail: Richard.guy@pharm.unige.ch			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The long-term objective is to develop and optimize a novel, noninvasive, iontophoretic approach for metabolic monitoring via the skin. The low-level current density drives both charged and highly polar (yet neutral) compounds across the skin at rates much greater than passive diffusion. As the skin offers a uniquely accessible body surface across which information can be extracted, we hypothesize that truly noninvasive and highly sensitive devices, which exploit uniquely paired flows of at least two substances, can be developed for iontophoretic monitoring applications. Proof-of-principle targets two analytes of significant interest; specifically, glucose and lactate.				
14. SUBJECT TERMS Metabolic monitoring; transdermal monitoring; glucose monitoring			15. NUMBER OF PAGES 92	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	10
Reportable Outcomes.....	11
Conclusions.....	13
Appendices.....	14

SKIN BIOENGINEERING: NONINVASIVE TRANSDERMAL MONITORING

Richard H. Guy, University of Geneva, School of Pharmacy, Switzerland

Introduction

The long-term objective was to develop and optimize a novel, noninvasive, iontophoretic approach for metabolic monitoring via the skin. The low-level current density drives both charged and highly polar (yet neutral) compounds across the skin at rates much greater than passive diffusion. As the skin offers a uniquely accessible body surface across which information can be extracted, we hypothesized that truly noninvasive and highly sensitive devices, which exploit uniquely paired flows of at least two substances, could be developed for iontophoretic monitoring applications. The research strategy attempted to optimize iontophoretic and sensing technology to satisfy three key criteria for success: (a) fundamental understanding of electrotransport across the skin; (b) reproducible enhancement of transdermal permeability to identify metabolic monitoring opportunities via the skin; and (c) characterization and validation of simple, user-friendly devices for sample collection coupled with sensitive and specific analytical tools. The specific aims of the project were:- {1} To refine understanding of electrotransport across the skin; to exploit the interactions (and independence) of solute and ion flows in the presence of an applied electric field. {2} To demonstrate that the simultaneous, 'reverse iontophoretic' extraction of a target analyte, together with an endogenous substance of essentially constant concentration within the body, can offer truly noninvasive, metabolic monitoring. {3} To engineer simple, elegant, prototypical devices, of small volume (100 μ L or less), into which reverse iontophoretically extracted samples may be efficiently collected. {4} To couple these systems to highly sensitive and specific chromatographic and electrochemical analytical tools both off-line and, eventually, on-line, *in situ*. Proof-of-principle targeted two analytes of significant interest; specifically, glucose and lactate. Furthermore, the bioengineering and analytical chemistry advances envisaged should allow broad, 'mass-screening' of the substances extracted (and extractable) by reverse iontophoresis revealing additional opportunities for the approach. In summary, this project aimed to evaluate iontophoretic bioengineering technology *in vivo* in man; specifically, applications with respect to metabolic monitoring have been examined.

Body

This project has resulted in a significant body of work, comprising:

{A} Two peer-evaluated reviews, one which summarizes the application of reverse iontophoresis for noninvasive transdermal monitoring in general, the other which specifically focuses on noninvasive and minimally invasive methods for transdermal glucose monitoring.

Review 1: Reverse Iontophoresis for Non-Invasive Transdermal Monitoring. B. Leboulanger, R.H. Guy and M.B. Delgado-Charro. *Physiol. Measure.* **25**: R35-R50 (2004).

Iontophoresis is the application of a small electric current to enhance the transport of both charged and polar, neutral compounds across the skin. Manipulation of either the total charge delivered and/or certain electrode formulation parameters allows control of electromigration and electroosmosis, the two principal mechanisms of transdermal iontophoresis. While the approach has been mainly used for transdermal drug delivery, "reverse iontophoresis", by which substances are extracted to the skin surface, has recently been the subject of considerable effort. Glucose monitoring has been extensively studied and other applications, including therapeutic drug monitoring, are contributing to the development of the technique. An internal standard calibration procedure may ultimately render this novel monitoring technique completely non-invasive.

Review 2: Noninvasive and Minimally Invasive Methods for Transdermal Glucose Monitoring. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Diabet. Tech. Therap.*, **7**: 174-197 (2005).

Noninvasive and minimally invasive techniques for monitoring glucose via the skin are reviewed. These approaches rely either on the interaction of electromagnetic radiation with the tissue or on the extraction of fluid across the barrier. The structure and physiology of the skin make the technical realization of transdermal glucose monitoring a difficult challenge. The techniques involving transdermal fluid extraction circumvent and/or compromise the barrier function of skin's outermost and least permeable layer, the stratum corneum, by the application of physical energy. While sonophoresis and microporation methods, for example, are in relatively early-stage development, a device using reverse iontophoresis [the GlucoWatch® Biographer (Cygnus, Inc., Redwood City, CA)] is already commercially available. Optical techniques to monitor glucose are truly noninvasive. The tissue is irradiated, the absorbed or scattered radiation is analyzed, and the information is processed, to provide a measure proportional to the concentration of glucose in the dermal tissue. These techniques include near-infrared and Raman spectroscopy, polarimetry, light scattering, and photoacoustic spectroscopy. By contrast, impedance spectroscopy measures changes in the dielectric properties of the tissue induced by blood glucose variation. Large-scale studies in support of efficacy of these methodologies are as yet unavailable. At present, therefore, transdermal fluid extraction technologies are offering greater promise in terms of practical and realizable devices for patient use. The truly noninvasive allure of the optical approach assures continued and

intense research activity—for the moment, however, an affordable, efficient and portable system is not on the immediate horizon.

{B} Four peer-reviewed articles, describing (i) proof, *in vitro*, of the internal standard concept as a noninvasive method for glucose monitoring by reverse iontophoresis; (ii) application of the internal standard concept to noninvasive glucose monitoring by reverse iontophoresis *in vivo*; (iii) the phenomenon of electroosmosis in transdermal monitoring and its implications for noninvasive and calibration-free glucose monitoring; and (iv) the simultaneous extraction of glucose and urea by reverse iontophoresis *in vivo*.

Article 1: Reverse Iontophoresis for Noninvasive Glucose Monitoring: The Internal Standard Concept. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *J. Pharm. Sci.*, **92**: 2295-2302 (2003).

Reverse iontophoresis is used by the GlucoWatch® Biographer to non-invasively extract glucose across the skin, allowing a diabetic's glycemia to be evaluated every 10 minutes over several hours. However, before each use, the device must be calibrated with a blood sample assayed in the conventional way. The objective of this study was to identify an approach by which to avoid this invasive step. The dermal (anodal) side of porcine skin *in vitro* was bathed in buffered (pH 7.4) solutions containing glucose, at concentrations from 3 to 10 mM, and physiological levels of sodium chloride. Constant current was applied and the cathodal solution contacting the outer skin surface was analysed periodically for the quantities of Na⁺ and glucose extracted by "reverse" electromigration and electroosmosis, respectively. While the extracted Na⁺ flux was invariant, as expected given the essentially fixed NaCl concentration present in the physiological system, the glucose samples reflected proportionately the subdermal concentration. Equally, the extracted flux ratio (glucose/sodium) varied linearly with the subdermal glucose/sodium concentration ratio; knowing the gradient of this correlation, therefore, means that a measurement of the extraction flux ratio can be used to determine the subdermal glucose concentration (the physiological [Na⁺] being known and fixed). Thus, a refinement of the reverse iontophoresis technology using the simultaneous determination of the extracted fluxes of the analyte of interest (glucose) and of an "internal standard", whose level in the biological system is invariant (Na⁺), may permit a noninvasive sampling methodology free of the need for calibration with a blood sample.

Article 2: Non-invasive Glucose Monitoring by Reverse Iontophoresis *In Vivo*: Application of the Internal Standard Concept. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Clin. Chem.*, **50**: 1383-1390 (2004).

The GlucoWatch Biographer uses reverse iontophoresis to extract glucose across the skin to monitor glycemia in diabetes. The invasive daily calibration with a conventional "fingerstick" has been perceived as a disadvantage. Here, an "internal standard" is used so as to render the approach completely non-invasive. The simultaneous extraction of glucose and sodium by reverse iontophoresis was performed on human volunteers over 5 hours, and blood glucose was measured in the conventional manner at each collection interval. These data were used for each subject to calculate an extraction constant (K),

which equals the ratio of the extracted fluxes ($J_{\text{glc}}/J_{\text{Na}^+}$) normalized by the corresponding ratio of the concentrations in the blood ($[\text{glu}]/[\text{Na}^+]$). The values of K were compared between and within subjects. The iontophoretically extracted glucose flux reflected the glucose concentration profiles in the blood, sodium extraction remained essentially constant consistent with the fact that its systemic concentration does not vary significantly. A constant value of K was established for 2/3 of the study population, therefore, allowing for an accurate prediction of glycemia without the need for a calibrating blood sample. However, further experimentation in additional subjects revealed seasonal changes in the efficiency of glucose extraction, that were not mirrored by the reverse iontophoresis of Na^+ . That is, variation in K became apparent. The "internal standard" might refine the determination of glycemia by reverse iontophoresis without calibrating with a blood sample. While Na^+ can be a useful internal standard for other applications, this research suggests that another neutral molecule, extracted via the same mechanism as glucose, would provide a better calibration.

Article 3: Electroosmosis in Transdermal Iontophoresis: Implications for Non-invasive and Calibration-free Glucose Monitoring. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Biophys. J.*, **87**: 3344-3350 (2004).

Reverse iontophoresis uses a small low electric current to non-invasively extract blood analytes, e.g. glucose, across the skin. The simultaneous quantification of the analyte extracted and of an additional endogenous substance of fixed and known concentration in the body, permits the blood level of interest to be found without the need for an invasive calibration procedure. The transport phenomena underlying this approach, applied to glucose monitoring, has been investigated *in vitro*, using Na^+ and neutral model solutes as endogenous "internal standards" (specifically, urea, glycerol, mannitol, and sucrose). The cathodal extracted fluxes of glucose under conditions of modified skin permselectivity were related to those of the different, potential "internal standards". Flux ratios depended upon the iontophoretic conditions and the size of the neutral "internal standards", while high variability was observed with Na^+ . Constant flux ratios were obtained with mannitol, glycerol, urea, and sucrose for which the mechanism of electrotransport was identical to that of glucose. The advantage of using a neutral internal standard, however, must be weighed against the need to identify and validate the marker under physiological conditions and the additional analytical chemistry necessary for the practical quantification of this substance.

Article 4: Simultaneous Extraction of Urea and Glucose by Reverse Iontophoresis *In Vivo*. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Pharm. Res.*, **21**: 1805-1810 (2004).

Reverse iontophoresis extracts glucose across the skin in the GlucoWatch Biographer[®], a device to monitor glycemia in diabetes. However, the device must first be calibrated with an invasive "fingerstick" and this has been perceived as a disadvantage. Here, urea, a neutral "internal standard" is extracted simultaneously in an attempt to render the technique completely non-invasive. In a 5-hour experiment in human volunteers, reverse iontophoretic fluxes of glucose and urea (J_{glu} and J_{urea} , respectively) were measured periodically and correlated with the corresponding blood levels. In the case of glucose, a finger-tip blood sample was taken at the beginning of each collection interval; for urea,

three blood samples were assayed – one before, one during, and one at the end of iontophoresis. The ratio $J_{\text{glu}}/J_{\text{urea}}$ divided by the ratio of the systemic concentrations ($C_{\text{glu}}/C_{\text{urea}}$) yielded an extraction coefficient (K) that could be compared between subjects. While J_{glucose} tracked C_{glu} faithfully when the volunteers were challenged with an oral glucose load, J_{urea} remained quite stable reflecting the fact that C_{urea} did not change appreciably during the experiment. However, while the variability (expressed as the coefficient of variation) in the normalized extraction flux of urea ($J_{\text{urea}}/C_{\text{urea}}$) was on the order of 25%, that for glucose was greater (>45%), with the result that the values of K (0.45 ± 0.25) were less constant than anticipated. Although urea performed quite reasonably as an internal standard, in that its extraction flux and systemic concentration both remained quite constant, the normalized, transdermal, iontophoretic flux of glucose showed inter-individual variability due to mechanisms that were not entirely governed by electrotransport. That is, despite good qualitative tracking to blood levels, there appear to be other (biochemical, metabolic, contamination?) factors that impact upon the quantitative results obtained.

{C} Abstracts describing initial efforts to use reverse iontophoresis for the noninvasive, transdermal monitoring of lactate. This work has been presented as posters at the 2004 Annual Meeting of the American Association of Pharmaceutical Scientists (Baltimore, MD), and at the 4th Annual Meeting of the Diabetes Technology Society (Philadelphia, PA). An article describing this work is currently being prepared for submission to *Diabetes Technology & Therapeutics*.

Non-invasive Monitoring of Lactate by Reverse Iontophoresis

S. Nixon¹, M. B. Delgado-Charro^{1,2}, and R. H. Guy^{1,2}.

¹School of Pharmacy, University of Geneva, Switzerland, and ²Department of Pharmacy and Pharmacology, University of Bath, UK.

Purpose: To apply transdermal reverse iontophoresis for non-invasive monitoring and clinical chemistry. L-lactate was studied because it serves as (i) a metabolic marker in the critically ill patient, and (ii) an indicator of performance in sports training.

Methods: *In vitro* iontophoresis experiments used pig skin. Current (0.4 mA) was applied for 5 hours. The subdermal solution was L-lactate (0.5-4.0 mM) in buffered NaCl at pH 7.4. Electrode solutions were 50 mM NaCl. L-lactate was quantified enzymatically. *In vivo*, two glass cells, containing NaCl, were attached to the forearms of 6 volunteers. Current (0.6 mA) was passed for 5 hours. Every 15 minutes, the anodal solution was removed for analysis and the cell refilled. Blood lactate was measured at each sampling interval.

Results: *In vitro*, passive lactate transport was negligible. A lactate reservoir existed in excised skin. Anodal extraction fluxes of lactate (J), once the skin reservoir was empty, correlated with subdermal concentrations (C); the extraction efficiency (J/C) was $9.4 (\pm 0.9)$ $\mu\text{L/h}$. *In vivo*, lactate extraction was facile and efficient. Again, a local reservoir, unrelated to systemic lactate, was observed but, once depleted, J/C values in 5 of 6 subjects were relatively stable: $\sim 61 (\pm 13)$ $\mu\text{L/h}$. However, towards the end of the experiment, in two subjects, and throughout for a final volunteer, J/C was significantly

higher: 133 (± 21) $\mu\text{L/h}$. Whether this is because immobilization of the subject's arm for several hours results in increased subdermal lactate at the iontophoresis site, or for another reason (e.g., contamination from sweat), requires further investigation.

Conclusion: The concept of non-invasive lactate monitoring by reverse iontophoresis is established. More work is required for optimization of the approach.

Funded by USAMRAA and NIH. The information presented does not necessarily reflect the position or the policy of the U.S. Government; no official endorsement should be inferred.

{D} Progress towards the 'engineering/analytical chemistry' objectives of the project has also been made (specifically: to engineer simple, elegant, prototypical devices, of small volume (100 μL or less), into which reverse iontophoretically extracted samples may be efficiently collected; and to couple these systems to highly sensitive and specific chromatographic and electrochemical analytical tools both off-line and, eventually, on-line, *in situ*). To-date, one publication has appeared:

Polyelectrolyte-modified short microchannel for cation separation. X. Bai, C. Roussel, H. Jensen and H. H. Girault. *Electrophoresis*. **25**: 931-935 (2004).

Three alkali cations, potassium, sodium, and lithium, have been separated within 15 s in a 1 cm long polymer microchip. The separation microchannel is modified by a polycation, poly(allylammonium chloride), which makes the channel surfaces positively charged leading to a reversed electroosmotic flow (EOF) when compared to bare channels. Due to the decreased apparent mobility of the cations, the separation resolution is improved allowing the use of shorter channels.

The electrochemical detection of glucose without mediator in a polymer microchip has also been accomplished (and was described in our previous annual report). An article describing this work is in preparation.

Key Research Accomplishments

- Establishment of proof-of-concept *in vitro* for an ‘internal standard’ approach in reverse iontophoretic monitoring.
- Demonstration *in vivo*, in man, that the internal standard concept can be applied to the noninvasive, reverse iontophoretic monitoring of glucose.
- Identification of significant inter- and intra-individual variability in electroosmotic flow induced by iontophoresis.
- Characterization of electroosmotic flow across the skin during iontophoresis, and elucidation of potential candidates for use as internal standards in the reverse iontophoretic monitoring of glucose.
- Illustration *in vivo*, in man, that urea may serve as a suitable internal standard for the reverse iontophoretic monitoring of glucose.
- Initial demonstration of the concept of reverse iontophoretic monitoring of lactate: *in vitro* and *in vivo* proof-of-concept.
- Electrochemical detection of glucose without mediator in a polymer microchip – towards a practical and efficient device for reverse iontophoretic monitoring.
- Development of a polyelectrolyte-modified short microchannel for cation separation, a potentially key component of a practical and efficient device for reverse iontophoretic monitoring.

Reportable Outcomes

Publications in peer-reviewed journals

Reverse Iontophoresis for Non-Invasive Transdermal Monitoring. B. Leboulanger, R.H. Guy and M.B. Delgado-Charro. *Physiol. Measure.* **25**: R35-R50 (2004).

Noninvasive and Minimally Invasive Methods for Transdermal Glucose Monitoring. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Diabet. Tech. Therap.*, **7**: 174-197 (2005).

Reverse Iontophoresis for Noninvasive Glucose Monitoring: The Internal Standard Concept. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *J. Pharm. Sci.*, **92**: 2295-2302 (2003).

Non-invasive Glucose Monitoring by Reverse Iontophoresis *In Vivo*: Application of the Internal Standard Concept. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Clin. Chem.*, **50**: 1383-1390 (2004).

Electroosmosis in Transdermal Iontophoresis: Implications for Non-invasive and Calibration-free Glucose Monitoring. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Biophys. J.*, **87**: 3344-3350 (2004).

Simultaneous Extraction of Urea and Glucose by Reverse Iontophoresis *In Vivo*. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Pharm. Res.*, **21**: 1805-1810 (2004).

Polyelectrolyte-modified short microchannel for cation separation. X. Bai, C. Roussel, H. Jensen and H. H. Girault. *Electrophoresis.* **25**: 931-935 (2004).

Meeting presentations, abstracts

Calibration-Free Glucose Monitoring Using Reverse Iontophoresis. A. Sieg, R.H. Guy and M.B. Delgado-Charro. 2003 Controlled Release Society 30th Annual Meeting Proceedings.

Non-invasive Monitoring of Lactate by Reverse Iontophoresis S. Nixon, M. B. Delgado-Charro and R. H. Guy. Presented as posters at the 2004 Annual Meeting of the American Association of Pharmaceutical Scientists (Baltimore, MD), and at the 4th Annual Meeting of the Diabetes Technology Society (Philadelphia, PA).

Simultaneous Extraction of Urea and Glucose by Reverse Iontophoresis *In Vivo*. A. Sieg, R.H. Guy and M.B. Delgado-Charro. Presented as a poster at the 4th Annual Meeting of the Diabetes Technology Society (Philadelphia, PA, 2004).

Thesis

Anke Sieg. "The internal standard concept for non-invasive glucose monitoring using reverse iontophoresis". Ph.D. thesis in Pharmaceutical Sciences awarded by the University of Geneva, Switzerland, 2004.

Conclusions

The long-term objective of this project was to develop and optimize a novel, noninvasive, iontophoretic approach for metabolic monitoring via the skin. The research has advanced iontophoretic and sensing technology and has addressed three key issues:

- (a) acquisition of a fundamental understanding of electrotransport across the skin;
- (b) demonstration of reproducible enhancement of transdermal permeability and identification of glucose and lactate as clear metabolic monitoring opportunities via the skin; and
- (c) development of practical devices for sample collection coupled with sensitive and specific analytical tools.

The work has refined our understanding of electrotransport across the skin, and has exploited the interactions (and independence) of solute and ion flows in the presence of an applied electric field. This has allowed the simultaneous, 'reverse iontophoretic' extraction of a target analyte, together with an endogenous substance (the "internal standard") of essentially constant concentration within the body. The approach can offer truly noninvasive, metabolic monitoring. In parallel, simple, elegant, prototypical devices, of small volume (100 μ L or less), into which reverse iontophoretically extracted samples may be efficiently collected, have been developed and tested. The manner in which these systems may be coupled to highly sensitive and specific chromatographic and electrochemical analytical tools has been demonstrated. Proof-of-principle targeted two analytes of significant interest; specifically, glucose and lactate.

In summary, this project aimed to evaluate iontophoretic bioengineering technology *in vivo* in man; specifically, applications with respect to metabolic monitoring of glucose and lactate have been shown.

Appendices

The following are appended as pdf files:

1. Reverse Iontophoresis for Non-Invasive Transdermal Monitoring. B. Leboulanger, R.H. Guy and M.B. Delgado-Charro. *Physiol. Measure.* **25**: R35-R50 (2004).
2. Noninvasive and Minimally Invasive Methods for Transdermal Glucose Monitoring. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Diabet. Tech. Therap.*, **7**: 174-197 (2005).
3. Reverse Iontophoresis for Noninvasive Glucose Monitoring: The Internal Standard Concept. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *J. Pharm. Sci.*, **92**: 2295-2302 (2003).
4. Non-invasive Glucose Monitoring by Reverse Iontophoresis *In Vivo*: Application of the Internal Standard Concept. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Clin. Chem.*, **50**: 1383-1390 (2004).
5. Electroosmosis in Transdermal Iontophoresis: Implications for Non-invasive and Calibration-free Glucose Monitoring. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Biophys. J.*, **87**: 3344-3350 (2004).
6. Simultaneous Extraction of Urea and Glucose by Reverse Iontophoresis *In Vivo*. A. Sieg, R.H. Guy and M.B. Delgado-Charro. *Pharm. Res.*, **21**: 1805-1810 (2004).
7. Polyelectrolyte-modified short microchannel for cation separation. X. Bai, C. Roussel, H. Jensen and H. H. Girault. *Electrophoresis*. **25**: 931-935 (2004).
8. Calibration-Free Glucose Monitoring Using Reverse Iontophoresis. A. Sieg, R.H. Guy and M.B. Delgado-Charro. 2003 Controlled Release Society 30th Annual Meeting Proceedings.
9. Non-invasive Monitoring of Lactate by Reverse Iontophoresis S. Nixon, M. B. Delgado-Charro and R. H. Guy. Presented as posters at the 2004 Annual Meeting of the American Association of Pharmaceutical Scientists (Baltimore, MD), and at the 4th Annual Meeting of the Diabetes Technology Society (Philadelphia, PA).
10. Simultaneous Extraction of Urea and Glucose by Reverse Iontophoresis *In Vivo*. A. Sieg, R.H. Guy and M.B. Delgado-Charro. Presented as a poster at the 4th Annual Meeting of the Diabetes Technology Society (Philadelphia, PA, 2004).

TOPICAL REVIEW

Reverse iontophoresis for non-invasive transdermal monitoring

Benoît Le Boulanger^{1,2}, Richard H Guy^{1,2}
and M Begoña Delgado-Charro^{1,2}

¹ School of Pharmacy, University of Geneva, 30, Quai Ernest-Ansermet CH-1211 Geneva 4, Switzerland

² Centre international de recherche et d'enseignement ('Pharmapeptides'), F-74160 Archamps, France

E-mail: richard.guy@pharm.unige.ch

Received 3 October 2003, accepted for publication 6 January 2004

Published 20 April 2004

Online at stacks.iop.org/PM/25/R35 (DOI: 10.1088/0967-3334/25/3/R01)

Abstract

Iontophoresis is the application of a small electric current to enhance the transport of both charged and polar, neutral compounds across the skin. Manipulation of either the total charge delivered and/or certain electrode formulation parameters allows control of electromigration and electroosmosis, the two principal mechanisms of transdermal iontophoresis. While the approach has been mainly used for transdermal drug delivery, 'reverse iontophoresis', by which substances are extracted to the skin surface, has recently been the subject of considerable effort. Glucose monitoring has been extensively studied and other applications, including therapeutic drug monitoring, are contributing to the development of the technique. An internal standard calibration procedure may ultimately render this novel monitoring technique completely non-invasive.

Keywords: iontophoresis, electromigration, electroosmosis, glucose monitoring, therapeutic drug monitoring

1. Introduction

Iontophoresis involves the application of a small and defined electrical current to the skin. This process causes increased molecular transport through the skin and has found application, therefore, in transdermal drug delivery (Burnette 1989). The concept is not new, however; the basic mechanisms were clearly appreciated by Leduc (Leduc 1900, Sage and Riviere 1992) in 1900. Nevertheless, it is only now that approved iontophoretic drug delivery systems are finally reaching the market (Merino *et al* 1997).

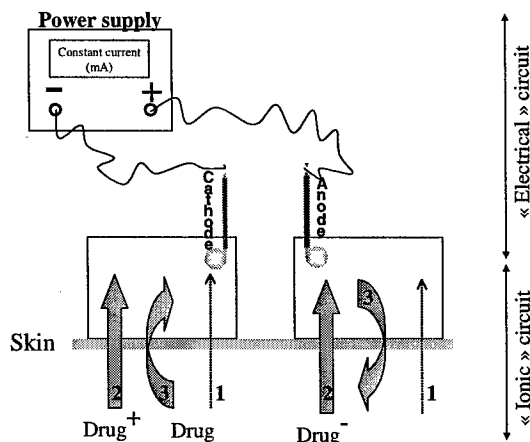


Figure 1. Reverse iontophoresis: a schematic diagram illustrating the experimental set-up. Constant current is delivered to the anode and the cathode from a power supply. Cationic and neutral substances are drawn towards the cathode by electromigration (2), electroosmosis (3) and (to a negligible extent, typically) passive diffusion (1). Anionic compounds are attracted into the anode chamber by electromigration (2), while convective solvent flow (3) opposes this phenomenon (again, passive diffusion (1) is negligible).

(This figure is in colour only in the electronic version)

The symmetry of iontophoresis means that it also enables extraction of solute molecules from within the subdermal compartment to the skin surface. The potential exists, therefore, to use the technique for clinical chemistry without blood sampling. Applications that may be envisaged include general blood chemistry, glucose monitoring, the detection of diagnostic markers and therapeutic drug monitoring (Merino *et al* 1997). The mechanism of extraction involves either electromigration of charged species to the electrode of opposite polarity, or electroosmosis of polar, neutral or zwitterionic, molecules to the cathode; hence for cations, both mechanisms are operative.

A number of excellent reviews on iontophoresis and its applications have been published (Burnette 1989, Sage and Riviere 1992, Pikal 1992, Ledger 1992, Phipps and Gyory 1992, Scott *et al* 2000, Delgado-Charro and Guy 2001). Here, attention is focused on reverse iontophoresis and its applications in diagnosis and monitoring. After a brief consideration of the basics underlying electrotransport across the skin, a detailed evaluation of the relevant literature and a perspective on the future are presented.

2. Mechanisms of transport during reverse iontophoresis

2.1. Electromigration

Conventionally, in iontophoresis, a constant current is applied, such that the flow of electrons is translated into an ion flux across the skin. A power supply establishes the electric field that causes electrons to migrate in the 'electrical' portion of the circuit and ions to flow in the 'ionic' part (figure 1). It follows that the number of electrons flowing through the 'electrical' portion of the circuit is exactly balanced by the amount of ionic charge flowing through the skin (Sage and Riviere 1992).

The electromigration contribution to iontophoretic transport is a direct result of current application. Ionic transport proceeds through the skin to maintain electroneutrality (Burnette 1989, Sage and Riviere 1992, Phipps and Gyory 1992). Faraday's law applies to steady-state transport and relates the number of ions crossing the membrane to the electric current, the time of current passage and the charge per ion (Burnette 1989, Sage and Riviere 1992):

$$M_i = \frac{Ti_i}{Fz_i} \quad (1)$$

where M_i is the number of moles of the i th ion, T is the time (s), z_i is the valence, F is Faraday's constant ($96\,487\text{ C mol}^{-1}$) and i_i is the current (A) carried by the i th species.

Given that usually there is more than one ion moving across the barrier, the total number of moles transported (M) by the total current flowing (I) is given by

$$M = \sum_i M_i = \frac{T}{F} \sum_i \frac{i_i}{z_i} \quad (2)$$

where

$$I = \sum_i i_i. \quad (3)$$

This leads to the concept of an ion transport number (efficiency of transport) which is the fraction of the total charge that it transports:

$$t_i = \frac{i_i}{I}. \quad (4)$$

It follows that equation (1) may be rewritten as

$$M_i = \frac{t_i IT}{Fz_i}. \quad (5)$$

With respect to the subject of this review, the experimentally measured extraction flux (J_i , mol s^{-1}) is defined by the ratio of the number of moles transported (M_i) to the sampling time (T) (i.e. the duration of reverse iontophoresis):

$$J_i = \frac{M_i}{T} = \frac{t_i}{Fz_i} I. \quad (6)$$

Equation (5) shows that iontophoretic extraction is determined by the intensity of current, the time of iontophoresis, the charge and the transport number of the ion of interest. Current intensity (I) is directly and easily controlled by the power supply but is limited, for practical purposes *in vivo*, to not more than 0.5 mA cm^{-2} (Ledger 1992). The time of each extraction period must be sufficiently long to ensure that enough analyte is available for detection but not so long that clinically significant changes in the systemic concentration may have occurred. In any case, it must be recognized that reverse iontophoresis can only provide an estimation of the average level of the analyte in the body during the sampling period.

The charge (z_i) of the extracted ion is dictated by its molecular structure, and determines the polarity of the electrode at which sampling/analysis will be performed. The transport number, however, is difficult to estimate theoretically as it depends on the other ions contributing to the transport of charge across the skin. When an electric field is established across a membrane, ions on either side will migrate in the direction dictated by their charge. The speed of migration of an ion is determined by its physicochemical characteristics and the properties of the media through which the ion is moving (Sage and Riviere 1992, Phipps and Gyory 1992, Scott *et al* 2000, Phipps *et al* 1989). The sum of the individual ionic charges flowing across the skin must equal the number of electrons 'delivered' by the power supply; in other words, there is 'competition' among all the ions present to carry the charge.

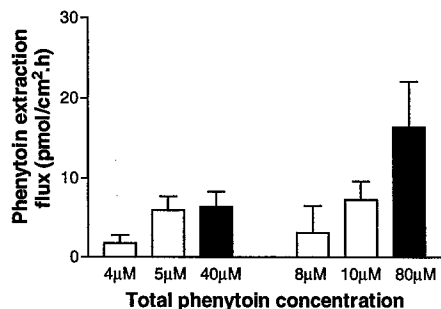


Figure 2. *In vitro* reverse iontophoresis fluxes of phenytoin at the anode illustrate that only free drug is extracted. The open bars (mean \pm SD) indicate extraction of drug from a subdermal compartment that did not contain albumin. The filled bars represent data obtained when human serum albumin was present at 44 g l^{-1} . The results are consistent with a free fraction of phenytoin of approximately 0.1. Data redrawn from Leboulanger *et al* (2003c).

The transport number of the ion of interest (the i th ion) may also be expressed as follows:

$$t_i = \frac{c_i z_i u_i}{\sum_{j=1}^n (c_j z_j u_j)} \quad (7)$$

where c_j is the concentration (mol cm^{-3}), z_j is the valence and u_j is the mobility ($\text{cm}^2 \text{ s}^{-1} \text{ V}^{-1}$) of each of the ' n ' ions in the system (Phipps and Gyory 1992).

Logically, the transport number depends on concentration (i.e. the available amount of a particular ion to participate in carrying charge across the skin). Likewise, it makes sense that ions, which are more mobile, will play a greater role in the movement of charge through the barrier. Note, however, that the relevant values of concentration (c) and mobility (u) are those inside the skin, rendering their estimation tricky at best and emphasizing the limitations of equation (7) as a predictive tool.

In reverse iontophoresis, the concentration of the analyte is the variable of interest and will depend (in the case of a drug being monitored, for example) on the dosage regimen and the relevant pharmacokinetics. Furthermore, with respect to electromigration, only the ionized fraction of the analyte is extractable and this will depend on the relevant pK_a . Similarly, for analytes that are bound to proteins, it is clear that only the free fraction can significantly contribute to charge transport across the skin. Figure 2 illustrates this point for phenytoin, a drug that is normally $\sim 90\%$ bound to albumin. As far as ionic mobility is concerned, regardless of the medium through which transport is occurring, an inverse dependence upon molecular size can be confirmed (and this is another reason, of course, why only unbound substances are extractable by reverse iontophoresis). In summary, therefore, it can be concluded that an ion can function as a major charge carrier if it is small, fully charged, at high concentration, and not significantly protein-bound. Additionally, and ideally, 'competing' ions are minimized, a situation not practically realizable for reverse iontophoresis where the major charge carriers are Na^+ and Cl^- .

Lastly, it is worth noting that the transport number is a 'formulation-dependent' parameter applicable for a given set of conditions. The transport number t_i can be determined experimentally; for example, by dividing the total amount of the ion transported by the total charge delivered (using Faraday's law, equation (1)), or from the gradient of a graph of ion flux versus current intensity (as in equation (6)) (Luzardo-Alvarez *et al* 2001).

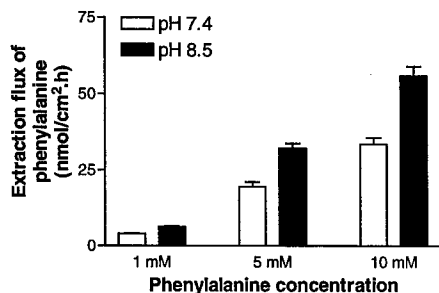


Figure 3. Electroosmotic extraction of phenylalanine as a function of (a) the subdermal concentration of the amino acid, and (b) the pH of the cathodal formulation. Reverse iontophoretic fluxes increased linearly with analyte concentration, and were significantly higher at pH 8.5 than at pH 7.4. Data redrawn from Merino *et al* (1999).

2.2. Electroosmosis

At physiologic pH, the skin is negatively charged and cation permselective. When an electric field is imposed across this type of membrane, there is convective or electroosmotic solvent flow induced in the anode to the cathode direction (i.e. in the direction of counter-ion migration) (Pikal 1990, 1992). This stream of solvent carries along with it dissolved solutes thereby enhancing the transport of neutral and, especially, polar molecules. Electroosmosis thus reinforces the transport of cations while acting against that of anions.

Important characteristics of this mechanism of electrotransport are, first, that the solvent volume flow (J_{vs} , volume \times time $^{-1} \times$ area $^{-1}$) is proportional to the potential gradient across the skin (Pikal 1990, Burnette and Ongpipattanakul 1987) and, second, that the electroosmotic flux of solute (J_i) is independent of molecular size (at least as long as the solute diameter does not approach that of the transport pathway) (Pikal 1990, 1992). The relationship between the molar flux (J_i) of the solute ' i ' and its molar concentration (c_i) is given by Pikal and Shah (1990):

$$J_i = J_{vs}c_i. \quad (8)$$

In an elegant series of experiments, J_{vs} during iontophoresis (Pikal and Shah 1990) was determined to be $6\text{--}19 \mu\text{l h}^{-1} \text{mA}^{-1}$.

In addition to the current density, the pH and the ionic strength are electrode formulation parameters that may modulate electroosmosis (Santi and Guy 1996a). Modifying the pH on either side of the skin can change the charge on the membrane and hence its permselectivity. Practically speaking, only the surface pH can be altered *in vivo*, of course. Figure 3 illustrates that cathodic extraction of phenylalanine is enhanced by as high a pH as can be feasibly maintained in contact with the skin surface (Merino *et al* 1999); in contrast, an acidic pH in the cathode chamber significantly impairs electroosmosis towards the electrode (Santi and Guy 1996a), while favouring extraction in the opposite direction, presumably due to a degree of neutralization of the fixed charge on the skin.

For cathodal extraction, electroosmotic flow is increased by lowering the ionic strength of the electrode formulation (Santi and Guy 1996a, Merino *et al* 1999). This phenomenon is less obvious for anodal extraction (Santi and Guy 1996a). However, it should be remembered that a finite level of electrolyte must be present in the electrode chambers (particularly at the anode) to support the Ag/AgCl electrochemistry.

It has also been found that an anode formulation with CaCl_2 or MgCl_2 , instead of NaCl , increased electroosmotic flow from beneath the skin surface towards the anode (Santi and Guy 1996b). Shielding of the net negative charge on the skin is a possible mechanism for this observation. In contrast, in the cathode chamber, to which electroosmosis predominates, enhanced solvent flow was achieved by formulating the electrode bathing solution with Ca^{++} binding agents (calcein, heparin or EDTA) presumably exposing a greater negative charge on the skin (Merino *et al* 1999, Santi and Guy 1996b).

2.3. The dominant mechanism: electromigration or electroosmosis?

For small mobile ions, electromigration is clearly the principal mechanism; similarly, for neutral, polar substances, electroosmosis dominates as there is no electromigration possible from the electrode. Both mechanisms of electrotransport depend upon the applied current (Phipps *et al* 1989, Padmanabhan *et al* 1990), with the effect being less marked for electroosmosis (Burnette and Ongpipattanakul 1987, Delgado-Charro and Guy 1994). As the size of an ion increases, its mobility is reduced and electromigration is compromised. For cations, this means that the dominant mechanism switches from electromigration to electroosmosis with increasing molecular size (Guy *et al* 2001); for anions, on the other hand, the two contributions will ultimately self-cancel and no transport will be observed. A weak acid, therefore, which is only partially ionized at physiological pH, may be more easily extracted as its neutral form to the cathode (e.g., theophylline (Glikfeld *et al* 1989, Sekkat *et al* 2002)).

3. Advantages and limitations of reverse iontophoresis

Non-invasive sampling methodologies are of obvious benefit to all patients for at least the following reasons: more information is obtainable as sampling can be performed more frequently, decreased pain and discomfort (i.e. better compliance), decreased risk of infection, potential for home-monitoring, etc.

Furthermore, there are special populations for whom non-invasive diagnosis and monitoring would be particularly useful: patients who are repetitively subjected to invasive blood withdrawal procedures, patients who over- or under-respond to standard therapeutic regimens, subjects who are least able to tolerate, recognize or communicate problems with unexpected drug effects and those who, for whatever reason, are either over- or under-dosed. Such patient populations include the 'critically' ill under intensive care, cancer and AIDS sufferers, pregnant women, those displaying unusual pharmacokinetics, patients receiving simultaneous, multi-drug dosing regimens (e.g., the elderly) and, of obvious special concern, are pediatric patients, for whom the need for non-invasive diagnosis and monitoring is particularly acute.

Even though reverse iontophoresis is much more efficient and reproducible than passive extraction, the quantities of analyte obtained at the skin surface are necessarily small. Dilution factors are likely to fall in the 10–100-fold range, or higher. Thus, analytical chemistry demands are significant and sampling periods may be so long, as a result, that changes in systemic concentration can occur. While this would be unacceptable in the case of glucose, for therapeutic drug monitoring at 'steady-state' the problem is less important (i.e. continuous monitoring is unnecessary, and a prolonged sampling time to obtain an average measure of concentration would be reasonable). Thus, an iontophoretic patch could be worn for a few hours at home, for example, and then sent to the clinical chemistry laboratory. Such an

Table 1. *In vivo* reverse iontophoretic extraction of potassium and sodium ions as a function of different parameters. The study group comprised 98 human volunteers. Data from Benjamin *et al* (1954).

	Potassium ($\mu\text{mol h}^{-1}$)	Sodium ($\mu\text{mol h}^{-1}$)	Ratio
Measurement time (10 measures)	1.8 ± 0.3	4.3 ± 0.5	2.4 ± 0.6
Measurement site			
Volar surface forearm	1.8 ± 0.4	4.3 ± 0.8	2.4 ± 0.4
Back of lower leg	1.6 ± 0.5	4.2 ± 0.8	2.7 ± 0.5
Upper part of abdomen	1.9 ± 0.4	4.5 ± 1.0	2.4 ± 0.5
Ambient temperature			
32–33 °C	1.8 ± 0.3	4.3 ± 0.5	2.4 ± 0.4
21 °C	1.7 ± 0.4	4.4 ± 0.6	2.5 ± 0.4
Gender			
Male	1.7 ± 0.5	4.3 ± 0.8	2.5 ± 0.3
Female	1.7 ± 0.5	4.2 ± 0.6	2.5 ± 0.3
Age			
<24 years	1.8 ± 0.5	4.2 ± 0.7	2.3 ± 0.5
25–49 years	1.7 ± 0.3	4.3 ± 0.5	2.6 ± 0.3
>50 years	1.6 ± 0.3	4.4 ± 0.5	2.7 ± 0.4

'off-line' analysis and quantification would be acceptable, and a sampling device simpler and less expensive to design.

An important limitation occurs when the skin accumulates the analyte of interest such that the initial extraction sample contains mostly information about this local 'reservoir' (this is the case for glucose (Glikfeld *et al* 1989) and lithium (Leboulanger *et al* 2003a)). A 'warm-up' period is necessary, therefore, before readings reflective of systemic levels are obtained.

It is also true that the reverse iontophoretic flux does not reach a constant, 'steady-state' rate instantaneously (Delgado-Charro and Guy 2003, Leboulanger *et al* 2003c)—the time to do so depends on the molecule of interest, and the dominant mechanism of electrotransport. However, whether this limitation is significant has not been completely established; for certain analytes, an acceptable correlation between extraction flux and subdermal concentration is also obtained prior to steady-state.

An additional, and significant, limitation is that reverse iontophoresis will simply not work for molecules with particular physicochemical properties. Specifically, proteins, for example, are simply too large to be extracted in amounts that are quantifiable. Extremely lipophilic compounds, with extremely small aqueous solubilities, will also be undetectable; unfortunately, cholesterol falls into this category.

4. Case studies

In 1954, reverse iontophoresis was first applied to the extraction of sodium and potassium ions (Benjamin *et al* 1954). Experiments were performed *in vivo*, in man, using a metal plate as the electrode. A current density of 0.5 mA cm^{-2} was applied over a skin surface of 8.3 cm^2 for 5 min or longer. In a total of nearly 100 subjects, it was shown that age, gender, measurement time, measurement site and ambient temperature did not significantly affect the amounts of the cations extracted (table 1). However, in the longer duration experiments, skin 'damage' was observed due to the fact that the pH of the cathodal solution increased from between 6

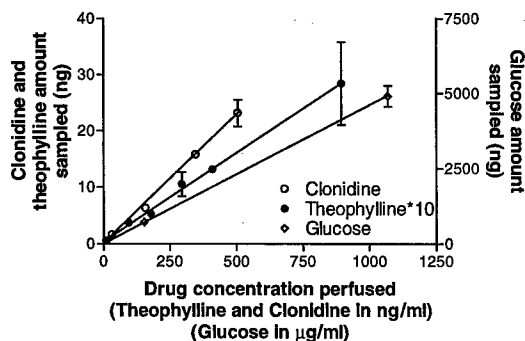


Figure 4. *In vitro* iontophoretic sampling of clonidine, theophylline and glucose. The amounts of the three compounds extracted across hairless mouse skin were linearly correlated with their subdermal drug concentrations. Data redrawn from Glikfeld *et al* (1989).

and 7 to nearly 11. Clearly, therefore, electrolysis of water was taking place at the bare metal electrode and, as a result, it became sensible to henceforth use electrochemically reversible electrodes (e.g., Ag/AgCl) in iontophoresis studies (Cullander *et al* 1993).

The practical potential of reverse iontophoresis was appreciated much later (in 1989) when it was demonstrated that the amount of a substance extracted across the skin in this way was linearly related to the subdermal (and, by extrapolation, the systemic) concentration (Glikfeld *et al* 1989). This relationship was shown for clonidine, theophylline and glucose; that is, for a more or less fully charged cation, for a partially charged anion and for a neutral polar molecule (figure 4).

Subsequently, attention was focused on glucose. Obviously, the availability of a non-invasive tool with which to monitor blood sugar in diabetics would be of immense medical benefit. The conventional, 'finger-stick' method, while precise and effective, is rarely used with sufficient frequency to reduce or avoid either hypo or hyper-glycaemic events, despite compelling evidence that such an approach can significantly impact the chronic progression of the disease (The Diabetes Control and Complications Trial Research Group 1993). Reverse iontophoresis experiments *in vitro* (Rao *et al* 1993) and initial *in vivo* studies in non-diabetic subjects (Rao *et al* 1995) established proof-of-concept, and led to the commercial development of an integrated device (the Glucowatch Biographer® (Cygnus, Inc. 2002, Tierney *et al* 2000b)) which is able to extract glucose iontophoretically across the skin and then assay sugar *in situ* with an on-board amperometric biosensor (figure 5). The mechanism of electrotransport of glucose is electroosmosis, meaning that, during each sampling period (20 min, initially, a shorter time in the G2 version of the device), the amount of analyte to be detected is very small. An exquisitely sensitive analytical method is therefore required, and involves a highly optimized adaptation of the Pt-glucose oxidase sensor (Tierney *et al* 1999).

A significant quantity of data has now been published to illustrate the efficiency of this reverse iontophoresis technology to track changes in the blood sugar levels of diabetics over the entire range of glycaemia (Tierney *et al* 2000b, Tamada *et al* 1995, 1999 Garg *et al* 1999, Tierney *et al* 2001, Pitzer *et al* 2001, Potts *et al* 2002). The quality of this information led to the Glucowatch being approved for use in adults by the U.S. Food and Drug Administration in 2001. Additional work in children (7–17 years) allowed this approval to be extended to juvenile diabetics the following year (Eastman *et al* 2002).

The long-term use and usefulness of the Glucowatch remain to be seen; nevertheless, there can be little doubt that this first truly non-invasive approach to the monitoring of blood

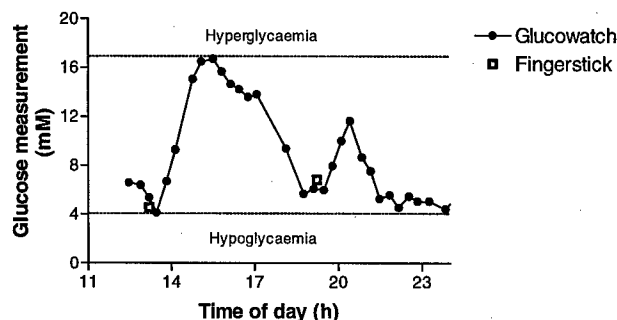


Figure 5. Continuous glucose monitoring *in vivo* with the Glucowatch Biographer® over a 12-h period (closed circles) compared with the typical information available to a diabetic from two 'finger-stick' measurements (open squares) pre-lunch and pre-dinner. Data redrawn from Tierney *et al* (2000b).

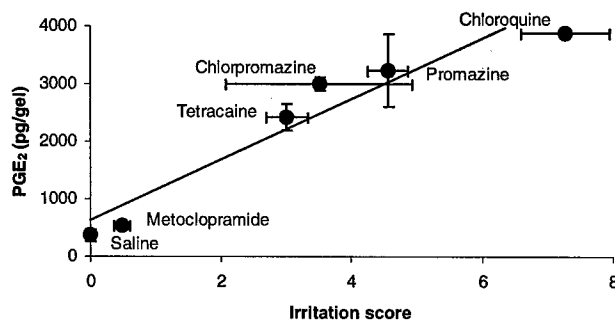


Figure 6. Correlation between PGE₂ efflux and irritation ($r^2 = 0.96$). Iontophoretic systems containing 100 mM drug were applied at $50 \mu\text{A cm}^{-2}$ for 24 h. Graph redrawn from Mize *et al* (1997).

sugar has made a paradigm shift in the field. It should be said that limitations of the approach are apparent, not least the lengthy, 2–3 h, warm-up time before measurements can be made (due to the need to empty a glucose reservoir in the skin) and the fact that a 'finger-stick' blood measurement is essential to calibrate the device.

Other applications of reverse iontophoresis can be divided into diagnosis/monitoring and therapeutic drug monitoring. An innovative concept was to use the approach as a diagnostic tool for cutaneous inflammation (Mize *et al* 1997). Prostaglandin E₂ (PGE₂) was monitored in response to the transdermal delivery of irritant drugs. It was hypothesized and shown that low-level iontophoresis (0.05 mA cm^{-2} over 2 cm^2) of saline did not by itself provoke an increased production of inflammatory markers *in vivo*, in the hairless guinea pig. Subsequently, potentially irritant drugs (chlorpromazine, chloroquine, promazine, tetracaine and metoclopramide) were administered iontophoretically (figure 6). Then, the anodal extraction of PGE₂ from the site of drug administration was monitored and compared to the saline control. Significant increases were observed that correlated well with more classic determinations of irritation (e.g., the Draize test, lesion score).

The reverse iontophoretic extraction of phenylalanine has also been demonstrated (Merino *et al* 1999). In phenylketonuria, a severe metabolic disease, the enzyme which

biotransforms phenylalanine is missing. Early detection of the disease and subsequent control of the diet are therefore essential. Children with the disease are frequently monitored via blood samples and a non-invasive approach would therefore be of interest. Phenylalanine is zwitterionic at physiologic pH and is therefore extracted during reverse iontophoresis by an electroosmotic mechanism. Like glucose, it has been shown that the amounts detected at the cathode are proportional to the subdermal concentrations (figure 3). However, phenylalanine systemic concentrations, even in phenylketonuria, are much less than typical glucose levels in diabetics. It follows that the analytical chemistry challenge for the monitoring of phenylalanine via reverse iontophoresis is considerable. On the other hand, it should be said that continuous and frequent monitoring, as performed by the Glucowatch®, is not necessary for sufferers of phenylketonuria; a device that simply collects the sample (once a day or once a week, for example), which is subsequently sent for analysis at a central analytical laboratory, would be perfectly acceptable.

Very recently, the reverse iontophoretic extraction of urea has been performed in 17 patients (21–35 years) with impaired kidney function (Degim *et al* 2003). Urea was extracted by electroosmosis to the cathode by current application for 5 min. The extracted amounts correlated well with urea levels in the blood ($r^2 = 0.88$). A logical application of this approach is to determine when dialysis should be performed in pediatric patients with kidney disease. A proof-of-concept study was subsequently conducted in six juvenile subjects (aged 9–16 years) for whom it was clearly shown that the amounts of urea extractable pre- and post-dialysis were quite different.

Therapeutic drug monitoring applications of reverse iontophoresis have recently attracted heightened interest. The potential of the approach was first explored using caffeine and theophylline in a model designed to mimic the developing cutaneous barrier in a premature neonate (Sekkat *et al* 2002). While the idea appears feasible for full-term infants, whose stratum corneum performs as well as that of an adult, the technique is less satisfactory when the barrier is impaired (as is the case, of course, for premature babies). The problem is caused by the fact that, superimposed upon the electrotransport of the target analyte being extracted by reverse iontophoresis, there is a significant passive transport which confounds straightforward interpretation of the data. Nevertheless, the non-invasive nature of the technique implies that it may have other useful applications in the case of the sick neonate, at least when skin barrier function is intact.

An important consideration when assessing the feasibility of reverse iontophoresis for therapeutic drug monitoring is the degree to which the compound of interest is protein-bound. Logically, only the free drug is electrotransported across the skin as the protein-bound form is too large to be extracted. This issue has been addressed with two anti-convulsant drugs, valproic acid (Delgado-Charro and Guy 2003) and phenytoin (Leboulanger *et al* 2003c), approximately 90% of which are typically bound to plasma proteins. Reverse iontophoresis extraction of both drugs in a concentration dependent fashion was demonstrated over a wide range encompassing those free levels observed in patients undergoing treatment. Valproate was extracted to the anode, while phenytoin ($pK_a = 8.3$) was recovered at both anode (the ionized fraction of the drug being attracted by electromigration) and cathode (the neutral form being carried by electroosmosis). When the level of protein was reduced in the subdermal compartment, the amount of drug extracted was increased consistent with the rise of the free drug level (figure 7). Equally, when monitoring phenytoin at a fixed subdermal protein level, introduction of valproate led logically to an increase in the free amount of the first drug and a higher rate of extraction due to the impact of competitive binding. The sensitivity of the method to respond to changes in free drug concentration in this way supports its potential usefulness for monitoring substances with a narrow therapeutic window. On the other hand, it must be

Phenytoin	80 μ M	40 to 80 μ M	80 μ M
Albumin	44 to 22 g/l	44 g/l	44 g/l
Valproate	-	-	0 to 542 μ M
Free Phenytoin	8 to 16 μ M	4 to 8 μ M	9 to 14 μ M

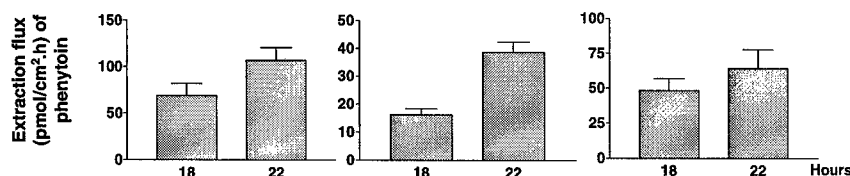


Figure 7. Monitoring of free phenytoin by reverse iontophoresis. The impact of (a) changing albumin concentration (1st panel), (b) changing drug concentration (2nd panel) and (c) addition of a competing drug, valproate (3rd panel) is illustrated. Data from Leboulanger *et al* (2003c).

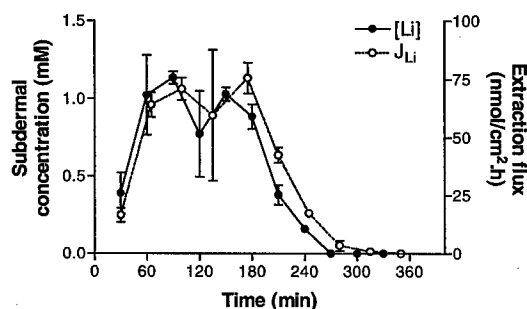


Figure 8. Monitoring of subdermal lithium concentration changes. Continuous line is the subdermal lithium concentration (mM). Dotted line is the cathodal extraction flux (nmol cm⁻² h⁻¹). Data taken from Leboulanger *et al* (2003b).

recognized that, for lipophilic drugs such as valproic acid and phenytoin, the free systemic concentrations are quite low (50–105 μ M and 4–8 μ M, respectively) and the amounts extracted by reverse iontophoresis are extremely small (indeed, the *in vitro* experiments described above were performed with radiolabelled drugs). Once more, the analytical challenge *in vivo* will be very demanding.

With lithium, a drug used to treat bipolar disorders, on the other hand, the analytical chemistry problem is much less severe. First of all, as a small, non-protein bound cation, Li⁺ is reverse iontophoretically extracted much more efficiently than the aforementioned anti-convulsant drugs (Leboulanger *et al* 2003b). Second, the effective plasma concentrations are much higher for lithium such that the amounts detected at the skin surface can be assayed with existing technology. *In vitro*, the linearity and rapidity of Li⁺ extraction from a physiological buffer were simply demonstrated (figure 8); when the concentration of Li⁺ in the sub-dermal compartment was varied over time, to simulate a pharmacokinetic profile, the extraction profile closely followed the 'absorption' and 'elimination' phases of the curve. Subsequently, an *in vivo* study (Leboulanger *et al* 2003a) in patients being treated with lithium has shown the potential of reverse iontophoresis to provide a useful clinical tool. The reverse iontophoretic extraction fluxes were extremely well correlated with the corresponding plasma concentrations.

5. Optimization

To expand the range of reverse iontophoresis applications, and improve on the existing technology, three strategies present themselves immediately for consideration.

Most obviously, an improvement in analytical sensitivity is desirable. The lower the extracted amount that can be detected, the larger the number of potential candidates for the technique, and the shorter the time of sampling (and, hence, the lower the total charge passed across the skin). Further discussion of this point, however, is outside the scope of this review.

Second, we may anticipate that optimization of the iontophoretic conditions will lead to maximization of the extracted amount. Thus, choosing the right current, current density, current profile and 'acceptor' phase for the extracted analyte may be expected to improve the extraction efficiency. It is well established that iontophoretic transport is directly proportional to the current and to the time of current application. In practice, however, there are limits to which these parameters can be increased. In terms of current density, it is generally agreed that 0.5 mA cm^{-2} is the maximum tolerable in man (Ledger 1992). It follows that the total current can be increased by increasing the surface area extracted. But, if one maintains the current density fixed, even under these conditions, the degree of sensation experienced by the subject increases with treated area, presumably because a greater number of dermal nociceptors are activated (Rao *et al* 1995). There are at least two other difficulties associated with increasing the total current and the area of skin over which reverse iontophoresis extraction is performed: (i) more current means that the (typically) Ag/AgCl electrodes used must be coated with an augmented layer of AgCl to ensure that the correct electrochemistry operates throughout the sampling period, and (ii) more area implies a greater volume into which the sample is collected, and this may place more demands on the analytical method. While it has been suggested that iontophoresis-induced reddening of skin can be reduced by 'pulsing' the current on/off at different frequencies (Santi and Guy 1996a), the practical result is that the impact of irritation is not proved. In addition, as the extraction is much less efficient during the 'off' period, the total time for sampling has to be increased so that the total desired current can be passed. Alternating current shows no benefits whatsoever; however, switching electrode polarity at the end of each sampling period, as is done in the Glucowatch®, has the distinct advantage of allowing regeneration of the Ag/AgCl electrodes (Tierney *et al* 2000a).

The third strategy is a method to avoid the present necessity to calibrate reverse iontophoretic extraction of an analyte with a blood sample. The amount of the compound of interest recovered at the skin surface is diluted in a certain volume of 'acceptor' fluid. The concentration therein depends on the efficiency of extraction (the analyte's transport number for an ion, the electroosmotic flow for a neutral species) and the volume of the 'acceptor' solution. In the case of the Glucowatch®, for example, in each sampling period, the electroosmotic flow of less than $1 \mu\text{l}$ is diluted into a volume of $400 \mu\text{l}$ (Tierney *et al* 2000a); that is, a three order of magnitude dilution of the glucose. Calibration is therefore essential to relate the amount of sugar extracted to the blood concentration. The concept of an 'internal standard' was initially proposed in 1993 (Numajiri *et al* 1993) and has recently been significantly refined and reduced to practice (Delgado-Charro and Guy 2003). The idea is as follows: reverse iontophoresis extracts numerous compounds at the same time; i.e. the process is non-specific and is only rendered specific for a particular compound by using a selective and precise assay. Suppose now that, in addition to the chosen target analyte (A), a second substance is also specifically analysed in each sampling period. Suppose, further, that the blood concentration of this second molecule (IS) is effectively constant. It follows that there should be a proportionality between the measured extraction flux ratio of A and

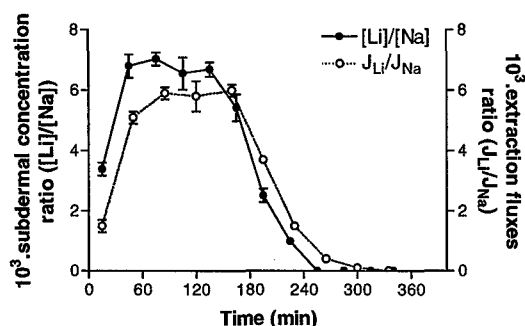


Figure 9. Simultaneous monitoring of lithium and sodium fluxes as a function of time. Data taken from Le Boulanger *et al* (2003b).

IS (J_A/J_{IS}) and the ratio of their subdermal, or blood, concentrations (C_A/C_{IS}):

$$\frac{J_A}{J_{IS}} = K \frac{C_A}{C_{IS}}. \quad (9)$$

It follows that, given C_{IS} is fixed and (presumably) known,

$$C_A = \frac{C_{IS}}{K} \frac{J_A}{J_{IS}}. \quad (10)$$

Thus, if the proportionality constant K can be determined and shown to be invariant in a subject population, then an experimental determination of J_A/J_{IS} , together with the known, 'constant' term (C_{IS}/K), allows C_A to be found without the need for blood sampling. The success of this idea rests on the independence of the iontophoretic transport of the analyte and the internal standard. In other words, it is important that a change in the transport number of A (due, for example, to a fall in its systemic concentration) is not compensated by an increase in that of IS. In this case, the validity of equation (9) breaks down. When the concept was proposed initially (Numajiri *et al* 1993), the design of the experiments led to exactly this violation, with the result that the method could not be validated. Recently, however, the principle has been re-visited (Delgado-Charro and Guy 2003), the experimental test designed more carefully, and the technique has been shown to work. The first demonstrated success involved the reverse iontophoretic extraction of valproate using glutamic acid as an anionic internal standard (Delgado-Charro and Guy 2003). Although the concentration of glutamate *in vivo* is not sufficiently constant for this amino acid to be considered as a practical internal standard, it served perfectly to prove the concept in this study. It was shown that (i) the extraction flux of valproate varied linearly with its subdermal concentration, (ii) the extraction flux of glutamate remained constant as the valproate concentration fluctuated and (iii) the ratio of the valproate to glutamate extraction fluxes was proportional both to their subdermal concentration ratio (equation (9)) and, as the glutamate concentration was fixed, to the valproate concentration. The approach allowed experimental variability to be reduced and permitted the subdermal valproate to be found even before the iontophoretic transport achieved 'steady-state'.

Subsequently, similar success was achieved with the Li^+/Na^+ (analyte/internal standard) couple (figure 9). The sodium ion is a useful and practical internal standard due to the fact that the concentration of NaCl *in vivo* does not vary outside the range of 125–145 mM (and typically remains within a much narrower window); Na^+ is also the major charge carrier in iontophoresis in the outward direction towards the cathode (much as Cl^- performs the same function towards the anode). *In vivo* measurements, in patients, confirmed the constancy of

the proportionality constant K in equation (9). However, while *in vitro* experiments (Sieg et al 2003) indicated that Na^+ may prove a valid internal standard for glucose also, a subsequent *in vivo* study revealed that electroosmotic flow is a much more sensitive phenomenon and can vary by nearly a factor of ten even while the electromigrative flux of Na^+ remains unchanged. It follows that it will be necessary to identify an electroosmotically-extracted internal standard for glucose in order to avoid the need for blood sampling.

6. Conclusion

Recent progress in reverse iontophoresis confirms its considerable potential. The approval and commercialization of the GlucoWatch® represents an important milestone for the technology as the first truly non-invasive monitoring device for diabetics. The value of the method is readily appreciated, furthermore, not only as a research tool but also as a practical means by which to improve the quality of care (and life) in patient populations for which repetitive blood sampling represents a significant burden: pediatric, geriatric and chronically ill individuals are obvious examples. Therapeutic drug monitoring is similarly accessible via reverse iontophoresis and it is hoped that the size of the commercial markets here (relative to that for glucose monitoring) does not deter the ultimate realization of practical devices. The future holds promise, in particular, if analytical tools continue to evolve in term of sensitivity, specificity and miniaturization, as they have in the recent past. If this is the case, then the application of reverse iontophoresis in 'smart', feedback drug delivery systems, and in remote sensing, can be foreseen.

Acknowledgments

This work was supported by the Fonds National Suisse de la Recherche Scientifique (3200-059042.99/1), the Programme Commun en Génie Biomédical of the EPFL and the Universities of Geneva and Lausanne and USAAMRAA grant DAMD 17-02-1-0712, Fort Detrick, MD. The information presented does not necessarily reflect the position or the policy of the U.S. Government, and no official endorsement should be inferred.

References

- Benjamin F B, Kempen R, Mulder A G and Ivy A C 1954 Sodium-potassium ratio of human skin as obtained by reverse iontophoresis *J. Appl. Physiol.* **6** 401-7
- Burnette R R 1989 Iontophoresis *Transdermal Drug Delivery* ed J Hadgraft and R H Guy (New York: Marcel Dekker) pp 247-91
- Burnette R R and Ongpipattanakul B 1987 Characterization of the permselective properties of excised human skin during iontophoresis *J. Pharm. Sci.* **76** 765-73
- Cullander C, Rao G and Guy R H 1993 Why silver/silver chloride? Criteria for iontophoresis electrodes *Proc. 3rd Int. Prediction of Percutaneous Penetration Conf. (La Grande Motte, France)* ed K R Brain, V J James and K A Watters pp 381-90
- Cygnus, Inc. 2002 *GlucoWatch: Automatic Glucose Biographer* <http://www.glucowatch.com>
- Degim I T, Ilbasimis S, Dundaroz R and Oguz Y 2003 Reverse iontophoresis: a non-invasive technique for measuring blood urea level *Pediatr. Nephrol.* **18** 1032-7
- Delgado-Charro M B and Guy R H 1994 Characterization of convective solvent flow during iontophoresis *Pharm. Res.* **11** 929-35
- Delgado-Charro M B and Guy R H 2001 Transdermal iontophoresis for controlled drug delivery and non-invasive monitoring *STP Pharm. Sci.* **11** 403-14
- Delgado-Charro M B and Guy R H 2003 Transdermal reverse iontophoresis of valproate: a non-invasive method for therapeutic drug monitoring *Pharm. Res.* **20** 1508-13

- Eastman R C *et al* 2002 Use of the GlucoWatch® biographer in children and adolescents with diabetes *Pediatr. Diabetes* **3** 127–34
- Garg S K, Potts R O, Ackerman N R, Fermi S J, Tamada J A and Chase H P 1999 Correlation of fingerstick blood glucose measurements with GlucoWatch Biographer glucose results in young subjects with type 1 diabetes *Diabetes Care* **22** 1708–14
- Glikfeld P, Hinz R S and Guy R H 1989 Noninvasive sampling of biological fluids by iontophoresis *Pharm. Res.* **6** 988–90
- Guy R H, Delgado-Charro M B and Kalia Y N 2001 Iontophoretic transport across the skin *Skin Pharmacol. Appl. Skin Physiol.* **14** 35–40
- Leboulanger B, Aubry J-M, Bondolfi G, Guy R H and Delgado-Charro M B 2003a Reverse iontophoretic monitoring of lithium *in vivo* *Proc. 8th Int. Cong. of Therapeutic Drug Monitoring & Clinical Toxicology, Ther. Drug Monit.* **25** 499
- Leboulanger B, Fathi M, Guy R H and Delgado-Charro M B 2003b Therapeutic drug monitoring by reverse iontophoresis *Proc. 30th Controlled Release Society Annual Meeting*
- Leboulanger B, Guy R H and Delgado-Charro M B 2003c Reverse iontophoretic monitoring of free phenytoin *Proc. 8th Int. Cong. of Therapeutic Drug Monitoring & Clinical Toxicology, Ther. Drug Monit.* **25** 499
- Ledger P W 1992 Skin biological issues in electrically enhanced transdermal delivery *Adv. Drug Deliv. Rev.* **9** 289–307
- Leduc S 1900 Introduction of medicinal substances into the depth of tissues by electric current *Ann. Electrobiol.* **3** 545–60
- Luzardo-Alvarez A, Delgado-Charro M B and Blanco-Mendez J 2001 Iontophoretic delivery of ropinirole hydrochloride: effect of current density and vehicle formulation *Pharm. Res.* **18** 1714–20
- Merino V, Kalia Y N and Guy R H 1997 Transdermal therapy and diagnosis by iontophoresis *Trends Biotechnol.* **15** 288–90
- Merino V, Lopez A, Hochstrasser D and Guy R H 1999 Noninvasive sampling of phenylalanine by reverse iontophoresis *J. Control Release* **61** 65–9
- Mize N K, Buttery M, Daddona P, Morales C and Cormier M 1997 Reverse iontophoresis: monitoring prostaglandin E2 associated with cutaneous inflammation *in vivo* *Exp. Dermatol.* **6** 298–302
- Numajiri S, Sugibayashi K and Morimoto Y 1993 Non-invasive sampling of lactic acid ions by iontophoresis using chloride ion in the body as an internal standard *J. Pharm. Biomed. Anal.* **11** 903–9
- Padmanabhan R V, Phipps J B, Lattin G A and Sawchuk R J 1990 *In vitro* and *in vivo* evaluation of transdermal iontophoretic delivery of hydromorphone *J. Control Release* **11** 123–35
- Phipps J B and Gyory J R 1992 Transdermal ion migration *Adv. Drug Deliv. Rev.* **9** 137–76
- Phipps J B, Padmanabhan R V and Lattin G A 1989 Iontophoretic delivery of model inorganic and drug ions *J. Pharm. Sci.* **78** 365–9
- Pikal M J 1990 Transport mechanisms in iontophoresis. I. A theoretical model for the effect of electroosmotic flow on flux enhancement in transdermal iontophoresis *Pharm. Res.* **7** 118–26
- Pikal M J 1992 The role of electroosmotic flow in transdermal iontophoresis *Adv. Drug Deliv. Rev.* **9** 201–37
- Pikal M J and Shah S 1990 Transport mechanisms in iontophoresis. III. An experimental study of the contributions of electroosmotic flow and permeability change in transport of low and high molecular weight solutes *Pharm. Res.* **7** 222–31
- Pitzer K R, Desai S, Dunn T, Edelman S, Jayalakshmi Y, Kennedy J, Tamada J A and Potts R O 2001 Detection of hypoglycemia with the Glucowatch biographer *Diabetes Care* **24** 881–5
- Potts R O, Tamada J A and Tierney M J 2002 Glucose monitoring by reverse iontophoresis *Diabetes Metab. Res. Rev.* **18** S49–53
- Rao G, Glikfeld P and Guy R H 1993 Reverse iontophoresis: development of a non invasive approach for glucose monitoring *Pharm. Res.* **10** 1751–5
- Rao G, Guy R H, Glikfeld P, LaCourse W R, Leung L, Tamada J, Potts R O and Azimi N 1995 Reverse iontophoresis: non invasive glucose monitoring *in vivo* in humans *Pharm. Res.* **12** 1869–73
- Sage B H and Riviere J E 1992 Model systems in iontophoresis—transport efficacy *Adv. Drug Deliv. Rev.* **9** 265–87
- Santi P and Guy R H 1996a Reverse iontophoresis—parameters determining electroosmotic flow: I. pH and ionic strength *J. Control Release* **38** 159–65
- Santi P and Guy R H 1996b Reverse iontophoresis—parameters determining electro-osmotic flow: II. Electrode chamber formulation *J. Control Release* **42** 29–36
- Scott E R, Phipps J B, Gyory J R and Padmanabhan R V 2000 Electrotransport systems for transdermal delivery: a practical implementation of iontophoresis *Handbook of Pharmaceutical Controlled Release Technology* ed D L Wise (New York: Marcel Dekker) pp 617–59
- Sekkat N, Naik A, Kalia Y N, Glikfeld P and Guy R H 2002 Reverse iontophoretic monitoring in premature neonates: feasibility and potential *J. Control Release* **81** 83–9

- Sieg A, Guy R H and Delgado-Charro M B 2003 Reverse iontophoresis for non-invasive glucose monitoring: the internal standard concept *J. Pharm. Sci.* **92** 2295–302
- Tamada J A, Bohannon N J V and Potts R O 1995 Measurement of glucose in diabetic subjects using noninvasive transdermal extraction *Nat. Med.* **1** 1198–201
- Tamada J A, Garg S, Jovanovic L, Pitzer K R, Fermi S, Potts R O and Cygnus Research Team 1999 Noninvasive glucose monitoring. Comprehensive clinical results *JAMA* **282** 1839–44
- The Diabetes Control and Complications Trial Research Group 1993 The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes-mellitus *N. Eng. J. Med.* **329** 977–86
- Tierney M J, Jayalakshmi Y, Parris N A, Reidy M P, Uhegbu C and Vijayakumar P 1999 Design of a biosensor for continual transdermal glucose monitoring *Clin. Chem.* **45** 1681–83
- Tierney M J, Kim H L, Burns M D, Tamada J A and Potts R O 2000a Electroanalysis of glucose in transcutaneously extracted samples *Electroanalysis* **12** 666–71
- Tierney M J, Tamada J A, Potts R O, Eastman R C, Pitzer K, Ackerman N R and Fermi S J 2000b The GlucoWatch® biographer: a frequent automatic and noninvasive glucose monitor *Ann. Med.* **32** 632–41
- Tierney M J, Tamada J A, Potts R O, Jovanovic L, Garg S and Cygnus Research Team 2001 Clinical evaluation of the GlucoWatch® biographer: a continual, non-invasive glucose monitor for patients with diabetes *Biosens. Bioelectron.* **16** 621–9

Review

Noninvasive and Minimally Invasive Methods for Transdermal Glucose Monitoring

ANKE SIEG, Ph.D.,^{1,2} RICHARD H. GUY, Ph.D.,^{1,3}
and M. BEGOÑA DELGADO-CHARRO, Ph.D.^{1,3}

ABSTRACT

Noninvasive and minimally invasive techniques for monitoring glucose via the skin are reviewed. These approaches rely either on the interaction of electromagnetic radiation with the tissue or on the extraction of fluid across the barrier. The structure and physiology of the skin make the technical realization of transdermal glucose monitoring a difficult challenge. The techniques involving transdermal fluid extraction circumvent and/or compromise the barrier function of skin's outermost and least permeable layer, the stratum corneum, by the application of physical energy. While sonophoresis and microporation methods, for example, are in relatively early-stage development, a device using reverse iontophoresis [the GlucoWatch® Biographer (Cygnus, Inc., Redwood City, CA)] is already commercially available. Optical techniques to monitor glucose are truly noninvasive. The tissue is irradiated, the absorbed or scattered radiation is analyzed, and the information is processed, to provide a measure proportional to the concentration of glucose in the dermal tissue. These techniques include near-infrared and Raman spectroscopy, polarimetry, light scattering, and photoacoustic spectroscopy. By contrast, impedance spectroscopy measures changes in the dielectric properties of the tissue induced by blood glucose variation. Large-scale studies in support of efficacy of these methodologies are as yet unavailable. At present, therefore, transdermal fluid extraction technologies are offering greater promise in terms of practical and realizable devices for patient use. The truly noninvasive allure of the optical approach assures continued and intense research activity—for the moment, however, an affordable, efficient, and portable system is not on the immediate horizon.

INTRODUCTION

DIABETES is a leading cause of death and disability in the world. A dramatically increasing number of people suffer from this

chronic disease, characterized by insulin deficiency and hyperglycaemia. The World Health Organisation expects the number of people with diabetes worldwide to reach 300 million by 2025.¹ In addition to life-threatening hypo-

¹School of Pharmacy, University of Geneva, Geneva, Switzerland.

²Rusham Park Technical Centre, Procter & Gamble, Egham, Surrey, United Kingdom.

³Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, United Kingdom.

The information presented does not necessarily reflect the position or the policy of the U.S. Government, and no official endorsement should be inferred.

and hyperglycaemic events, the disease presents long-term complications for the eyes, kidneys, peripheral nerves, heart, and blood vessels. Quick diagnosis and tight blood glucose control are therefore critical for the management of the patient with diabetes.

Clinical trials have confirmed that intensified insulin therapy significantly reduces the long-term complications of diabetes mellitus.²⁻⁵ However, such an approach also increases the risk of hypoglycaemic events, so that frequent and accurate glucose monitoring is mandatory if the disease is to be effectively brought under control. Conventional self-testing methods require a drop of blood for each glucose measurement, a painful and inconvenient procedure with poor patient compliance. Consequently, there has been, and continues to be, considerable investment in the development of continuous glucose monitoring technologies.⁶⁻¹³ A variety of techniques are being examined, such as the minimally invasive harvesting of interstitial fluid (ISF), the use of transdermal extraction, optical sensors, and the physical implantation of devices to measure glucose directly in a local subcutaneous environment.

This review focuses on non- and minimally invasive techniques for glucose monitoring that rely either on the interaction of electromagnetic radiation with the tissue or on the extraction of fluid across the skin.¹⁰ The state-of-the-art with respect to implantable glucose sensors and "ISF harvesting,"⁹ such as microdialysis or open-flow microperfusion, is covered elsewhere.^{6,11,14} Similarly, fluorescence-based sensors, which are generally coupled to

a fluid collection technique or an implant,⁷ are discussed elsewhere.¹⁵ A recent publication in this area reports the development of a potentially noninvasive glucose sensing contact lens.¹⁶

Of the more than 100 companies that are currently developing glucose monitoring devices, the most prominent examples, applying either transdermal fluid extraction or a specific, optical technique, are listed in Tables 1 and 2, respectively. In the following sections of this article these technologies are discussed in detail, and their potential is illustrated (whenever possible) by relevant data. First, though, the physiological constraints faced by transdermal and optical glucose monitoring approaches are considered.

T1,2

"PHYSIOLOGICAL ISSUES" FOR TRANSDERMAL AND OPTICAL GLUCOSE SENSORS

The skin as a glucose sensing site

The skin acts primarily as a barrier protecting the body from excessive water loss, from the entry of potentially hazardous substances, from cell damage due to ultraviolet radiation, and so on. It follows, therefore, that there are important anatomical and physiological features of the skin that render glucose monitoring across this organ a significant challenge.

The skin consists of two distinct components, the epidermis and the dermis, and rests on subcutaneous tissue consisting of fat and muscle (Fig. 1). The epidermis averages 0.1 mm in thickness and can be subdivided into several

F1

TABLE 1. TRANSDERMAL GLUCOSE MONITORING TECHNOLOGIES

Principle	Company	References	URL
Reverse iontophoresis	Cygnus	Rao et al., ¹⁷ Tamada et al., ¹⁸ Eastman et al., ¹⁹ Potts et al. ²⁰	www.glucowatch.com
Ultrasound	Sontra	Kost et al., ²¹ Mitragotri et al. ²²	www.sontra.com
Blister technique	ICB	?	www.glucose-monitor.com
Microneedles	Kumetrix	Smart et al., ²³ Smart and Subramanian ²⁴	www.kumetrix.com/technology.html
Altea MicroPor™ Laser	SpectRx	Gebhardt et al. ²⁵	www.spectrx.com/cgms.asp
Hypodermic needle	Integ	Stout et al., ²⁶ Collison et al. ²⁷	Currently unavailable

TABLE 2. NONINVASIVE OPTICAL GLUCOSE MONITORING TECHNOLOGIES IN DEVELOPMENT

Principle	Company	References	URL
Impedance spectroscopy	Pendragon Medical	Hayashi et al., ²⁸ Caduff et al., ²⁹ Pfützner et al., ³⁰	www.pendragonmedical.com
NIR	Sensys Medical	Malin et al., ³¹ Blank et al., ³² Ruchti et al., ³³	http://www.sensysmedical.com/home.html
NIR	InLight Solutions	Fleming et al., ³⁴ Robinson et al. ³⁵	www.inlightsolutions.com
NIR	VivaScan	Harjunmaa et al. ³⁶⁻³⁸	No website
NIR	Matsushita	Maruo et al. ^{39,40}	
NIR	Lifetrac Systems	?	www.sugartrac.com
NIR	CME Telemetry	Pawluczyk et al., ⁴¹ Samsoodar et al. ⁴²	www.cmetele.com
Kromoscopy	Optix Corp.	Sodickson and Block, ⁴³ Block et al. ⁴⁴	
NIR (thermal)	Abbott	Yeh et al. ⁴⁵	www.abbott.com
NIR (thermal)	Infratec	Malchoff et al. ⁴⁶	No website
MIR (thermal)	OptiScan	Zheng et al. ⁴⁷	No website
MIR	MedOptix	Berman and Roe ⁴⁸	www.medoptix.com
Raman	LightTouch	Chaiken and Peterson ^{49,50}	www.lighttouchmedical.com/plat.html
Scattering	Roche Diagnostics	Heinemann and Schmelzeisen- Redeker, ⁵¹ Heinemann et al. ⁵²	www.roche.com
Occlusion spectroscopy	Orsense	Cohen et al., ⁵³ Fine et al. ⁵⁴	www.orsense.com/prod.html
Polarimetry	TecMed	?	
Photoacoustic spectroscopy	Glucon	Pesach et al., ⁵⁵ Raz et al., ⁵⁶ Grinberg et al. ⁵⁷	www.tecmed.com www.glucon.com

Adapted from Koschinsky and Heinemann.⁶

layers: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. The outermost layer, the stratum corneum, is approximately 15–20 mm thick, and its cells are constantly being shed from the body. It consists of 15–25 dense layers of flattened, hexagonal corneocytes embedded in an intercellular lipid matrix. The lipophilic, dense nature of the stratum corneum is the principal obstacle to overcome when attempting to noninvasively sample glucose from the underlying tissue.⁵⁹

The epidermis is an epithelial membrane in a constant state of turnover. Glucose is the major substrate for metabolic activity, and glucose-metabolizing enzymes are present in the epidermis.⁶⁰ This layer of the skin is avascular and receives all nutrients via diffusion from the underlying dermis. Glucose is found in the interstitial space and intracellularly.⁶¹ Further, glucose is formed at the stratum granu-

solum-stratum corneum interface via the enzymatic breakdown of ceramides.⁶² It follows that attention must be paid when using a glucose monitoring technique that samples the analyte (all or in part) from the epidermis, as the correlation with the blood may be indirect.^{10,17,63}

The interface between epidermis and dermis is irregular and comprises a succession of finger-like projections called papillae (Fig. 1). The dermis is on the order of 1 mm thick and contains collagen, elastic fibers, blood vessels, and nerves, as well as hair follicles and sweat glands. The dermis is the usual site of glucose sensing, as home monitoring techniques take samples from dermal capillary blood; likewise, many new approaches are attempting to sample glucose within the dermal ISF. This deeper layer of the skin is supplied with glucose, oxygen, etc., from the systemic circulation. The

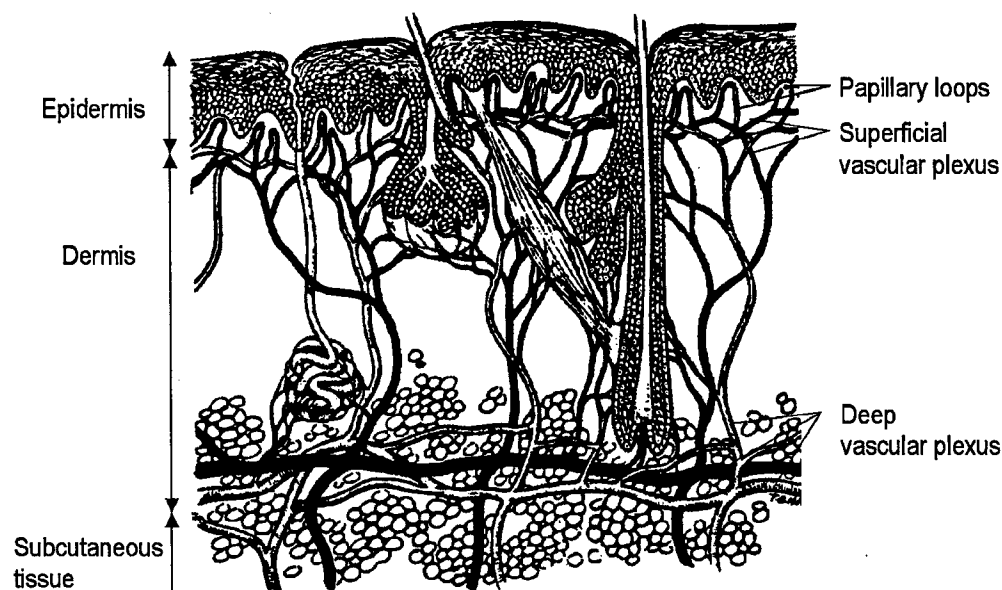


FIG. 1. Schematic diagram of the skin illustrating the microvasculature supply. Redrawn with permission from Yum and Roe.⁵⁸

dermal microcirculation comprises the deep vascular plexus at the interface between the dermis and the subcutaneous fat, the superficial vascular plexus located at 0.3–0.6 mm from the skin surface, and the small capillary loops that go into each dermal papilla tip less than a few hundred microns from the skin surface. The latter supply nutrients to the epidermis. The dilatation or constriction of skin blood vessels is controlled by the autonomic nervous system in response to stress, temperature change, and/or biochemical inputs. The skin microcirculation changes as well with age and disease state (e.g., the progression of diabetes), phenomena that may be expected to affect glucose measurements in the skin.^{10,58}

General optical properties of the skin

The optical properties of skin are governed by absorption and scattering, which combine in a complex fashion to attenuate light passing through the tissue.⁶⁴ A fraction of light traversing a tissue is not scattered; that is, the direction of propagation is not changed. In this case, Lambert-Beer's law is satisfied, and the transmission of the radiation depends on the tissue's absorption coefficient. Another portion of the light entering the tissue is scattered when

the size of scattering particles and the wavelength of light are of the same order of magnitude. Scattering originates primarily when light crosses the boundary between two media with different refractive indices. The scattering properties of a tissue can be expressed (similarly to absorption) in terms of a scattering coefficient. Together, the absorption and scattering coefficients determine an effective attenuation coefficient of light in a given tissue.^{13,64,65}

In broad terms, the stratum corneum and epidermis represent a light-absorbing layer, in that specific pigments are present for ultraviolet photoprotection. However, at wavelengths in the near-infrared (NIR), the transmittance of light through the stratum corneum and epidermis is 90–95% and is independent of skin pigmentation.⁶⁶ In the dermis, light scattering is more important. The most prominent scattering structures are believed to be collagen and elastic fibers, representing approximately one-half of the volume fraction. ISF accounts for about 90% of the rest, leaving only a small fraction occupied by cells and blood vessels.⁵¹ The latter are principally responsible for light absorption at wavelengths less than 600 nm. Beyond this threshold, a sharp decrease in absorption by hemoglobin and skin pigment, to-

gether with less dermal scattering, allows significant penetration of red light and NIR radiation. The most penetrating optical wavelength is 1,150 nm, at which about one-quarter of the incident radiation traverses the entire dermis (Fig. 2).⁶⁶ Thereafter, absorption by water becomes significant. The 600–1,300 nm region is therefore called “the optical window” of the skin, and NIR measurements have been the focus for the development of totally noninvasive glucose sensing approaches. This wavelength range, however, does not contain much specific information for glucose, and the NIR range above 1,300 nm, where specific glucose absorption bands are apparent, has become important, too. Between 1,520 and 1,850 nm, scattering predominates, and absorptions by water and fat are already significant. Between 2,000 and 2,500 nm, absorption dominates, with water, fat, and protein the primary absorbers.⁶⁷

Chemical, structural, and physiological variations, such as skin temperature, hydration state, or local skin blood flow, occur naturally and affect the tissue's optical properties and the depth of light penetration.³¹ Thus, the skin presents a real challenge to the development of reliable optical methods for glucose sensing.

Relationship between glucose concentrations in blood and ISF

Glucose monitoring has traditionally relied on blood levels. With the development of non-

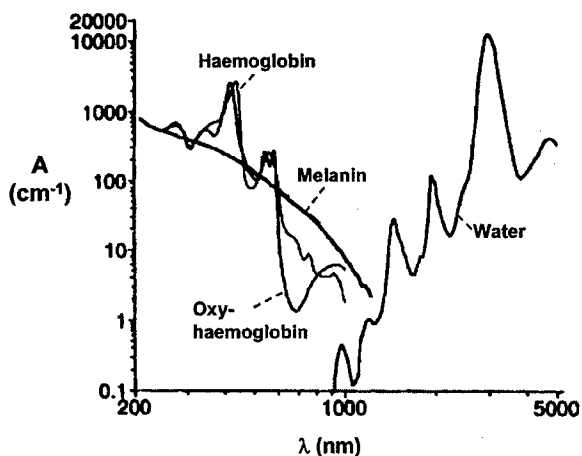


FIG. 2. Principal light-absorbing molecules in the skin. Redrawn from Anderson.⁶⁶ The most penetrating wavelengths are from 600 to 1,300 nm, the so-called “optical window” of the skin.

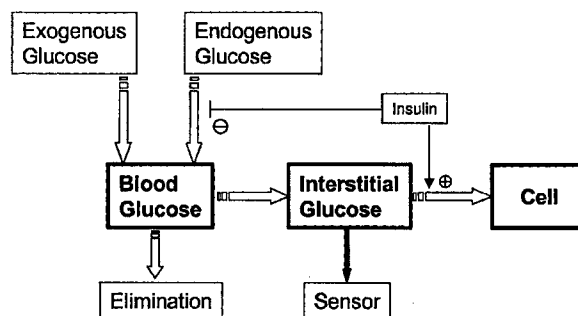


FIG. 3. A model for glucose sensing from ISF. The different glucose inputs and outputs affecting the measurement of glycaemia in the ISF relative to that in blood are highlighted. Redrawn with permission from Kulcu et al.⁷¹

and minimally invasive sensing approaches, other body compartments have been examined, with particular attention focussed on the ISF. The relevance of glucose levels measured in the ISF depends on the existence of an accurate, precise correlation between these values and those in the systemic circulation.¹⁰

The interstitium is a passive medium with no flow and little convection; the volume of this compartment between cells is very small. Glucose enters the ISF uniquely from the blood, and is removed primarily by uptake into the surrounding cells. Under physiological conditions, there is a free and rapid exchange of glucose between the plasma and the interstitial space, and changes in the concentration of glucose in the ISF are strongly correlated, therefore, with those in blood.⁶⁸ However, local changes in the blood flow or metabolic rate affect glucose levels in the ISF, such that changes in glucose levels here do not always mirror what is happening in blood. The lag time is reported to vary between 0 and 45 min^{26,68–72} (depending on the individual, the rate of glucose change, and the sampling methodology), and complicates interpretation of the measurement because the ISF glucose concentration does not always lag behind, but may also precede, the blood level (and/or change at a greater rate).^{68,71,73,74} Multicompartment models have been developed to explain this complex relationship^{68,71} (Fig. 3), and they underline that a rapid change in blood glucose (following an insulin injection, for example) will be reflected in the ISF with a delay. It follows that this phe-

nomenon can introduce an apparent error into the performance of a monitoring device that samples glucose in the ISF.⁷⁰

The relationship between glucose concentrations in blood and ISF is also important with respect to sensor calibration. All existing glucose sensing methods are calibrated against a measurement made from a conventionally obtained blood sample, and subsequent monitoring is based upon this calibration value. The clinical importance of the calibration is self-evident; if it is not accurate, all glucose measurements, hypo- and hyperglycemic alarms, adjustments to insulin dose, etc., would be based on a biased value.

Of course, the adjustment of therapy is traditionally based on blood glucose measurements, although it has been speculated that the ISF level might actually be more relevant because it is this fluid that bathes and feeds the cells.^{6,10} Clearly, while this issue falls outside the scope of the present review, it should be reiterated that the minimally and noninvasive approaches discussed below are sampling and monitoring glucose in the ISF, or a similar compartment, and not in blood. This renders evaluation of the technical performance of the different devices more difficult, as deviations from the reference value in the blood are not only generated by technical limitations, but may simply be due to physiological factors. The complex relationship between blood and the interstitial compartment requires, therefore, further elucidation—both in terms of optimizing metabolic control and in terms of the objective performance evaluation of different glucose monitoring devices.

TRANSDERMAL TECHNOLOGIES— FLUID EXTRACTION FROM SKIN

Transdermal techniques aim to access the ISF by the application of physical energy. A device is attached to the skin, either during or subsequent to the application of energy, and a sample, whose glucose concentration is directly related to that in the ISF, is then extracted.

Reverse iontophoresis

The GlucoWatch® Biographer (Cygnus, Inc., Redwood City, CA) uses reverse iontophoresis

to extract glucose across the skin. The technique is based on the application of a low electric current, which drives charged and uncharged, polar species across the skin at rates much greater than their passive permeabilities.⁷⁵ Electron flux from the external current source is transformed into the movement of ions via the electrode reactions. Ionic transport proceeds through the skin to maintain electroneutrality via electromigration. As the skin is negatively charged at physiological pH, it is permselective to cations. This preferential passage of positive counterions (mainly Na⁺) induces an electroosmotic solvent flow that carries neutral molecules in the anode-to-cathode direction (Fig. 4). By this mechanism, glucose is extracted towards the skin surface at a flux directly proportional to its concentration in the dermal ISF.^{17,18,76}

The latest version of this device, the GlucoWatch Biographer G2™, monitors glucose continuously for up to 13 h, recording six glucose readings per hour. Before each use, a 2-h "warm-up" period is required to remove residual glucose from the upper epidermal layers⁶² and to establish constant electroosmotic flow. The apparatus is then calibrated with a conventional blood sample from the fingertip to provide the proportionality constant necessary to convert the measured extraction flux into a systemic glucose concentration. During each measurement cycle, glucose is extracted iontophoretically for 3 min and is then quantified over the next 7 min during which no current is applied.⁷⁷ Electrode polarity is then re-

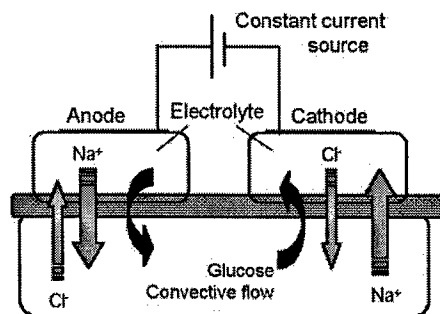


FIG. 4. Principle of glucose extraction by reverse iontophoresis. Preferential Na⁺ migration across the permselective membrane induces convective solvent flow, which carries glucose towards the cathodal chamber.

versed, and the 10-min cycle is repeated. [Note that reversal of the electrode polarity serves to maintain the Ag/AgCl iontophoresis electrodes.] The concentration of glucose achieved in the gel collection pads of the GlucoWatch are on the order of $1/1,000^{\text{th}}$ of that in the ISF, necessitating a very sensitive assay method. This is achieved via the reaction of glucose with the enzyme glucose oxidase and the subsequent electrochemical detection of the H_2O_2 formed on a platinum electrode. The skin's permselectivity and the characteristics of reverse iontophoresis allow several problems associated with the electrochemical detection of glucose using glucose oxidase to be avoided, including: (1) because of the low glucose amount extracted, the supply of oxygen is not a factor; (2) high-molecular-weight species, which may cause electrode fouling, are effectively filtered by the skin; and (3) electrochemically active substances, such as uric and ascorbic acids, are extracted towards the anode and do not interfere with the quantification of glucose at the cathode.⁷⁷ At present, the GlucoWatch is the only transdermal device (excluding implantable sensors that are inserted through the skin) that has received approval from the U.S. Food and Drug Administration. Its performance has been extensively evaluated in adults and children with diabetes and has shown adequate precision for home glucose monitoring.^{19,77-80} Figure 5 compares glucose concentration profiles from two subjects with the corresponding control (blood) measurements.

F5

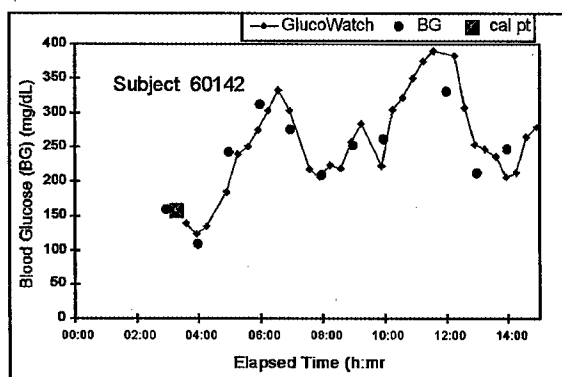


Figure 6 summarizes the data from 124 subjects on a Clarke Error Grid.⁸¹ Data points in the regions A and B are considered clinically acceptable, and represent 94.2% of all measurements ($r = 0.80$). The mean relative difference from the reference glucose value was 7.0%.

F6

Although the GlucoWatch represents a great technological achievement, its acceptance as a new tool for glucose monitoring has not been instantaneous for various reasons. To begin with, it is a complicated device that demands education of both patient and practitioner. It generates, furthermore, a lot of information, and much more than a typical patient with diabetes receives today; there is an issue, therefore, as to what should be done with all those data. The long warm-up time and the need for a blood calibration are additionally perceived as disadvantageous and detract from the user-friendliness of the system. The application of the current causes mild or moderate erythema that disappears relatively quickly. As well, the GlucoWatch cannot provide glucose monitoring when a patient is actively sweating; readings are "skipped" under these circumstances until the subject stops perspiring. There is also concern that the GlucoWatch is less useful for detecting hypoglycemic events than first envisaged.⁸² And, finally, this sophisticated technology comes at a price that, for the moment, is not cheap and is such that it has certainly deterred the more widespread adoption of the methodology.

As mentioned previously, the glucose analysis is based on the ISF concentration, and in-

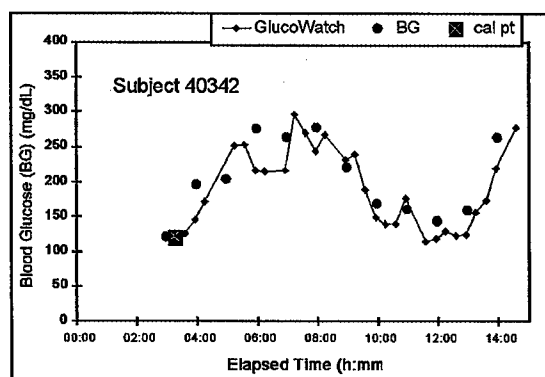


FIG. 5. Glucose monitoring with the GlucoWatch Biographer in a home environment. For the two subjects shown, all points lie in the clinically acceptable (A + B) region of a Clarke Error Grid. cal pt, calibration point. Redrawn with permission from Tierney et al.⁷⁷

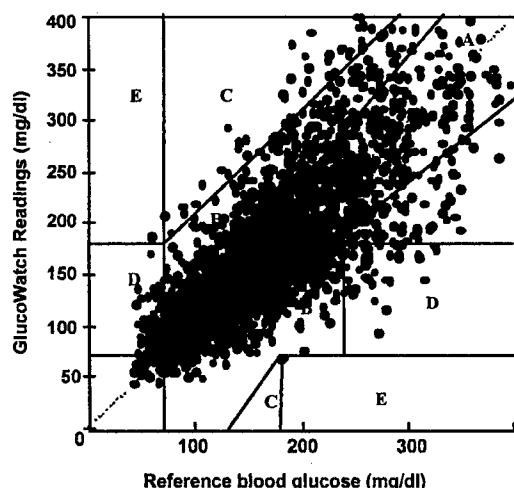


FIG. 6. Correlation of GlucoWatch Biographer readings versus reference blood glucose (home environment study). Data include 2,996 single readings from 124 subjects. The overall regression slope was 0.95, $r = 0.80$. Of the points, 94.2% lie in zones A + B. Data are from Tierney et al.⁷⁷

terpretation of a particular reading must therefore take into account the physiological delay between the blood and ISF compartments as well as an instrumental lag time resulting from the sample collection and analysis period.⁷¹ The newer GlucoWatch has an improved alarm function that additionally tracks the rate of change of glucose concentration so as to identify rapidly falling glucose levels before hypoglycaemia is reached.^{83,84} Additional work is examining whether the invasive calibration step can be avoided via the use of an "internal standard."⁸⁵⁻⁸⁷

Analogous to iontophoresis, enhanced glucose extraction has been attempted by generating an electroosmotic flow after current application via a modified skin suction system.⁸⁸ A macro-perforated cannula was inserted into the interstitial space to harvest ISF, and current was applied via integrated electrodes to increase the flow rate. However, the formation of bubbles in the tissue and local reactions to the implanted cathode hampered the establishment of a constant flow. It is not clear if this approach is currently being pursued.

Sonophoresis

In contrast to therapeutic ultrasound operating in the range of 0.7–3 MHz, low-frequency

ultrasound (~20–100 KHz is able to increase the skin's permeability such that drug delivery and glucose biosensing become feasible).⁸⁹ The progression of the ultrasonic longitudinal waves causes compression and expansion of the medium at the same frequency of the ultrasound applied, thus creating pressure variations. To allow ultrasound to penetrate into body tissues, a contact medium, such as an aqueous gel, is necessary because ultrasound is completely reflected by air. The impact of ultrasound on the biological tissue exposed is described by a relationship analogous to Lambert-Beer's law:

$$I(x) = I_0 \times \exp(-ax) \quad (1)$$

where I_0 and $I(x)$ are the intensities of ultrasound at the tissue surface and at a depth (x) into the tissue, respectively, and a is the absorption coefficient of the tissue. The principal effects of ultrasound are: (1) local heating, (2) cavitation, and (3) acoustic streaming. For low-frequency ultrasound applied to the skin, cavitation is believed to be the most important mechanism by which the barrier is compromised.⁹⁰ The expansion and subsequent collapse of gaseous inclusions in close proximity to the skin surface appear to create new pathways through the stratum corneum, presumably via the reversible disordering of intercellular lipid bilayers.⁹¹ The penetration enhancement is determined by the total energy applied, and is a function of intensity, duty cycle, application time, and frequency.

Glucose monitoring using sonophoresis has been investigated *in vivo* in rats^{21,22,92} and in patients with diabetes.²¹ To optimize glucose extraction in humans, ultrasound at 20 kHz was applied for up to 2 min using a coupling medium of phosphate-buffered saline containing 1% sodium lauryl sulfate. Glycemia was monitored for 4 h by periodically applying a vacuum to the treated area for 5 min. The extraction of ISF was reasonably efficient: on average, $25 \mu\text{L cm}^{-2} \text{h}^{-1}$ (that is, an order of magnitude greater than that achieved by reverse iontophoresis). Analysis of the extracted ISF and comparison of the glucose therein with simultaneously measured blood samples gave a mean relative error of 23%. After the

sonophoretic pretreatment, the skin's permeability to glucose remained elevated for 15 h but then returned to normal by 24 h. The level of enhancement induced varied by up to a factor of 10, suggesting that individual calibration would be necessary for the practical application of the approach.²¹

Recently, the sonophoretic technique has been refined such that the application of a vacuum is rendered unnecessary and extracted glucose is detected in situ.⁹³ The ultrasound application is now of short duration, and the use of lactic acid (a hyperosmotic agent) enables the outward flux of sufficient glucose that monitoring is possible. Data from 10 subjects were collected, and 60% of the transdermal glucose measurements correlated well with blood glucose ($r > 0.7$). Accuracy was reflected in a Clarke Error Grid (Fig. 7), with 92% of the measurements falling in the clinically acceptable A + B region. The method is reported to be painless with the use of a known irritant (sodium lauryl sulfate) and a known keratolytic agent (lactic acid) apparently not resulting in any untoward effects. Nevertheless, there remain long-term safety and efficacy studies to be performed, as well as optimization of the "hardware" and analytical chemistry required

for continuous monitoring. Finally, although the synergistic effects of sonophoresis with other skin penetration enhancement technologies (including iontophoresis, for example) has been reported in the literature,⁹⁴ the developmental challenge of such a combined approach has not yet been taken up—at present, such a strategy would be too complicated and too expensive to warrant serious consideration.

Skin suction blister technique

This technique uses a vacuum to form blisters, a few millimeters in diameter, at the epidermal-dermal junction⁹⁵; the dermis is left almost completely intact.⁹⁶ It is assumed that the fluid obtained from the vesicles formed is a mirror of the ISF.^{95,97,98} Biochemical analysis reveals a "serum-like" pattern qualitatively, although the protein content is lower.⁹⁹ The correlation between glucose in the blister fluid and that in the blood is once again an issue with this approach, therefore.¹⁰ Once a blister is formed, the vacuum is released, and a sample of the blister fluid is collected immediately for analysis. Repeated measurements with vacuum application for a defined time (typically 10–20 min) are possible during several days.⁹⁷ The technique has been reported to be painless and well tolerated, with minimal risk of infection. However, it is acknowledged that the suction procedure might cause cell damage as elevated levels of lysosomal and cytosolic enzymes have been found in the blister fluid relative to that in serum.⁹⁹

In a glucose clamp study in subjects with diabetes, glucose concentrations in the blister fluid were compared with those in plasma¹⁰⁰; blistering times were also determined. Blister concentrations were lower than plasma glucose, but a good correlation between the values was found. Further, longer blistering times were correlated with higher hemoglobin A_{1c} values. The lower glucose concentration in the blister was suggested to be due to glucose consumption by the filtration barrier (i.e., the dermal tissue). Further measurements were made in healthy volunteers and in patients with diabetes,^{97,101} using a device developed for practical use. Skin blisters were created over a 6-day period. Following an oral glucose tolerance

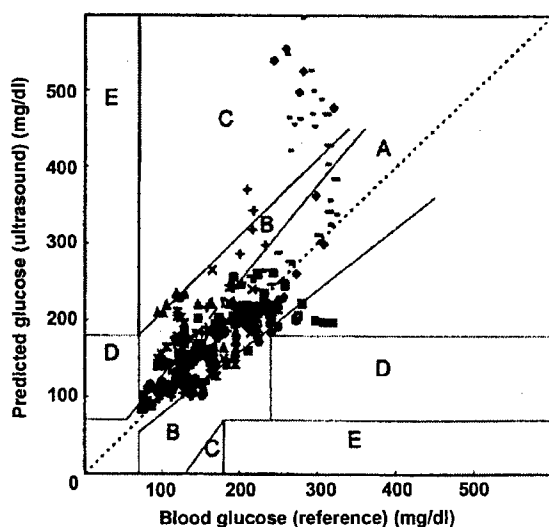


FIG. 7. Accuracy of glucose prediction from ultrasonically permeated skin. Data are from 10 subjects with diabetes. Of the points, 92% fall within the clinically acceptable A + B region. Redrawn with permission from Chuang et al.⁹³

F7

test (OGTT), the results implied that the glucose kinetics in the ISF were similar to those in capillary blood. Analysis of other metabolites (glycerol, 3-hydroxybutyrate, lactate) and insulin in the blister fluid was also performed.¹⁰¹ While good agreement between ISF and plasma concentrations for glycerol and 3-hydroxybutyrate was found, significantly higher lactate levels, and lower insulin concentrations, were measured in the skin blisters. Mild inflammation after formation of the erosion was reflected by increased exudation and higher extraction. However, no complications were reported, and epidermal regeneration was reported to be rapid. Clinical studies have been announced in which the technique will be evaluated in newborn infants.¹⁰¹ It must be stated, however, that this approach does not appear, at the moment, to be less invasive than a simple finger-prick measurement of blood glucose. It is necessary to create a skin erosion and then maintain that site open for a significant period so that systemic glucose levels can be measured. While this condition is tolerable in relatively short-duration experiments, it remains to be seen whether the chronic use of this technique in individuals with diabetes will ever be acceptable.

Micropores

Methods to perforate the stratum corneum, without penetrating the entire skin, attempt to

punch micro-pathways through the outermost part of the barrier. A transdermal glucose sensing system (developed by SpectRx) uses a pulsed laser beam to create micropores of $<100\ \mu\text{m}$ in diameter by thermal ablation of the stratum corneum.²⁵ Transdermal fluid is then collected, by applying a vacuum, into a disposable collection device; glucose is subsequently quantified electrochemically. In a study with 56 subjects with diabetes, good agreement between extracted glucose and its capillary concentration was observed over a 2-day period: 93% of the data points fell in zones A and B of a Clarke Error Grid (r for the complete data set unknown). Precision was hampered when fluid flow was low; temperature variation was reported as a source of error. The poration procedure was considered well tolerated, and vacuum suction yielded transdermal flow rates of $5\text{--}15\ \mu\text{L/h}$. Skin blisters formed over the suction area, but lesions disappeared within 1 week for all subjects. As for some of the other techniques mentioned above, there remain a number of safety and practicality issues to be resolved before this approach might be considered for long-term, chronic applications.

A novel bioMEMS device for noninvasive glucose sampling across the skin has been described.¹⁰² A polydimethylsiloxane-based skin patch is used to sample glucose after thermal ablation of the stratum corneum by locally applied micro-heaters (Fig. 8). The intensity and

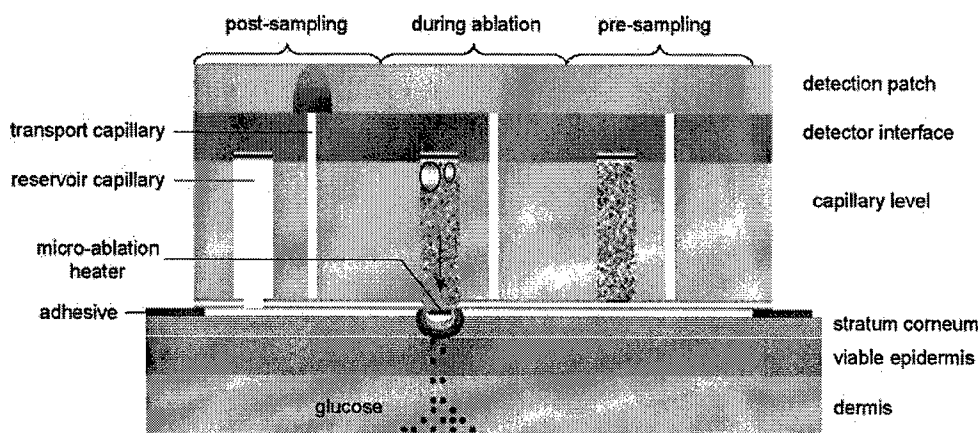


FIG. 8. The polydimethylsiloxane-based patch device is fixed to the skin surface. The stratum corneum is thermally ablated, allowing glucose to diffuse from the dermis into the capillaries of the device. The molecules reach the detection layer by capillary forces, where they are quantified colorimetrically. Redrawn with permission from Paranjape et al.¹⁰²

duration of heat application are controlled to ensure that no damage to living tissue or nerves occurs. Glucose then diffuses across the created micropores into the adhesive patch, which also comprises capillary channels for fluid transfer and a detector interface. Glucose is analyzed semiquantitatively by colorimetry. To date, however, the thermal ablation procedure has been tested only on reconstructed skin equivalents; safety, *in vivo*, has not been evaluated.

A related technology uses hollow microneedles.¹⁰³ An integrated microsampling and assay device uses a hollow silicon microneedle of 175 μm thickness to puncture the skin, allowing 200 nL of blood to be withdrawn into a microcuvette by capillary forces.¹⁰⁴ Glucose is assayed by glucose dehydrogenase-formazan dye chemistry. Biocompatibility and pain response were tested on patients with diabetes and healthy subjects, revealing that the minimally invasive procedure was virtually sensation-less and more comfortable than a conventional finger stick. Glass microneedles to extract ISF from the skin have also been developed,¹⁰⁵ and allow 1–10 μL of ISF to be collected via a multiple-needle array under negative pressure. Good correlation with capillary blood glucose has been reported in six subjects.

Overall, these microporation methods hold some promise, assuming that they can be reliably and economically fabricated. The issue of needle robustness, for example, needs to be resolved. Whether such an approach is best used continuously (via hollow structures) or via multi-application is not resolved and will certainly depend on numerous factors, including assay sensitivity, tolerability, cost of goods and manufacturing, and regulatory issues.

Other transdermal techniques

An integrated sampler has been developed in which a 30-gauge collection needle is connected to a glucose assay device.²⁶ The cannula penetrates the dermis to a controlled depth of less than 2 mm, and approximately 1 μL of dermal ISF is sampled. Glucose is then quantified by a standard glucose oxidase-mediated colorimetric measurement. The use of a hypodermic needle makes this approach quite invasive, despite claims that the technique is virtually

painless and avoids blood sampling. Glucose has also been measured transdermally by tape-stripping the stratum corneum and then drawing ISF to the skin surface by vacuum application.^{106,107} Glucose analysis involved an ion-sensitive field-effect transistor.¹⁰⁸ Even if such a method measures systemic glucose levels accurately, the approach is clearly not acceptable for chronic use in patients with diabetes.

RADIATION TECHNIQUES

Optical methods for glucose monitoring are considered to be truly noninvasive. A light beam interrogates the sample, and its interaction with the tissue, based on absorption and/or scattering, is used to quantify glucose. However, this apparently simple concept is complicated by the fact that the skin is a highly heterogeneous tissue, containing significant amounts of other substances (including proteins and water) that interfere with the measurement of glucose. Sophisticated, and typically expensive, equipment is necessary, therefore, (1) to record sufficient spectra and (2) to perform a detailed computational analysis of the data, so that a calibration model can be established. The multivariate chemometric methods used introduce a significant the risk of either overfitting the data or of finding a correlation between spectroscopic signal and glucose-specific information by chance. McNichols and Coté⁷ have described this problem aptly and stated that: "isolating those changes which are due to glucose alone and using them to predict glucose concentration is a significant challenge in itself, and to some extent all optical glucose sensing methods rely on solving the so-called 'calibration problem.'" This section illustrates these difficulties with a number of examples using "classical" NIR absorption or attenuated reflectance measurements.

Mid-infrared (MIR) and NIR absorption and reflectance measurements

Optical absorption spectroscopy for glucose quantification has generally been restricted to either MIR or NIR wavelengths. Figure 9 shows

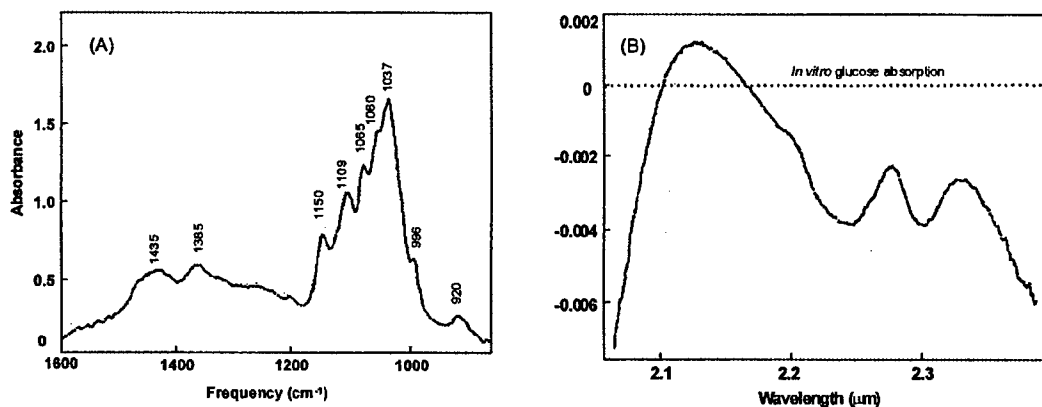


FIG. 9. Optical absorption spectra for glucose. A: MIR region extending from 1,600 to 900 cm^{-1} or 6.25 to 11 mm. B: NIR region extending from 2.0 to 2.5 mm or 5,000 to 4,000 cm^{-1} . Note that the magnitude of the three absorbance peaks in the NIR region is much smaller. Redrawn with permission from McNichols and Coté.⁷

MIR and NIR absorption spectra for aqueous glucose solutions after water subtraction. The selective absorption of light by a molecule is described by Lambert-Beer's law:

$$I = I_0 \times \exp(-\epsilon cl) \quad (2)$$

where I_0 is the intensity of the incident optical radiation, I is the transmitted intensity, ϵ is the molar absorptivity in $(\text{mol/L})^{-1} \text{cm}^{-1}$, c is the molar concentration, and l is the pathlength in cm. The molar absorptivity is fixed at each wavelength by the vibrational properties of the molecule. When attempting to determine glucose concentrations in the clinically pertinent range (2–22 mM) with all tissue components contributing to the background signal, the optical pathlength is an important parameter. That is, it must be long enough that a measurable glucose absorbance signal is produced, but should not be too long as this will increase noise and light attenuation by the tissue.⁶⁷

In the MIR region of the spectrum (2.5–50 μm , or 4,000–200 cm^{-1}), absorption bands due to fundamental stretching and bending modes of the molecule are observed. This so-called "fingerprint" region is commonly used, therefore, for compound identification. However, the high background absorption of other components in solution (especially water!) massively limits the useful pathlength range that can be used (to a few hundred micrometers or less), and little information on the commercial development of this approach is available.⁴⁸

MIR reflectance spectroscopy has also been used to measure glucose in aqueous solutions and in blood.⁷ In human whole blood samples, glucose has recently been quantified with a prediction error of 0.95 mM.¹⁰⁹ On the other hand, the feasibility of reflectance MIR in vivo, across the skin, has not yet been demonstrated.

The NIR region of the spectrum extends from 700 to 2,500 nm (14,000–4,000 cm^{-1}) and contains little specific information. Absorption bands are due to low-energy electronic vibrations (700–1,000 nm), and overtones of molecular bond stretching and combination bands (1,000–2,500 nm). Generally, only the first, second, and third overtones can be detected, and then only at high concentrations of the absorbing species, because intensity drops off rapidly with overtone order. The absorption bands are broad and easily influenced by hydrogen bonding and temperature effects, and show substantial overlap. NIR spectroscopy is therefore somewhat empirical and is not particularly suited for the identification of specific chemical species. On the other hand, NIR is attractive for quantitative spectroscopy since the spectra in that region are less affected by water, thereby allowing pathlengths of 1 mm to 1 cm to be used.⁷ Additionally, it is in this region that light penetrates on the order of centimeters into the skin, the so-called "optical window." Multivariate statistical techniques allow quantitative spectral analysis.

Glucose has been quantified in aqueous solutions, whole blood, and tissue phantoms, and

across the finger and other body parts (including the tongue⁶⁷), and this research has been extensively reviewed.^{7,8,12,13} There is significant commercial activity in the development of the NIR approach for noninvasive glucose monitoring (Table 2). Such development, however, is confronted with the significant challenge of producing a user-friendly, and ideally portable, device that works reliably *in vivo*. Making the transition from well-controlled model systems in the laboratory to real-world measurements on a fingertip or earlobe has not proved straightforward.⁸

F10

In addition, as shown in Figure 10, other substances in the body (proteins, urea, cholesterol, alcohols, etc.) have overlapping spectra with glucose and complicate the calibration process.⁴⁵ Blood flow and its pulsatile nature, furthermore, cause the optical pathlength and hence the measured glucose concentration to vary.^{10,51} In addition, the widely variable optical properties of tissue in the NIR, coupled with the fact that the glucose signal is low for concentrations in the physiological relevant range, remain an important challenge. Despite ongoing progress,^{32,39,110–112} therefore, the technology has a long way to go before it will provide reliable glucose monitoring devices for patient use.

Kromoscopy

An alternative method, called "Kromoscopy,"⁴³ is an analogue of human colour perception. Unlike the classical NIR approach

of analyzing spectra for glucose absorption at distinct wavelengths, kromoscopy works directly with the relative intensities of overlapping responses from four detectors, which record spectra over broad, different wavelength ranges. Signals are processed by vector analysis, which is reported to enhance the differentiation between analyte and interferences.⁴³ Recent work has shown that the magnitude of the kromoscopic signal is produced by "water displacement," whereby an increase in the glucose concentration yields a lower absorbance.¹¹³ This is due to the fact that an increased number of glucose molecules in the optical path means a greater displacement of water; as water has higher absorptivity than glucose, an increase in the level of sugar means a lower overall absorbance. However, water displacement is unspecific and makes calibration complicated.¹³ Nevertheless, urea and glucose have been distinguished in binary solutions,¹¹³ suggesting that the method may be feasible *in vivo*.¹¹⁴ Further work has discussed calibration *in vitro* and *in vivo* and has determined the influences of temperature, scattering, and other factors on kromoscopic measurements.^{115–118}

Thermal infrared measurements

The strong dependence of the infrared signal on temperature presents a significant source of error for reflectance measurements. However, this apparent thermo-optical response of the skin can also provide an opportunity. Indeed,

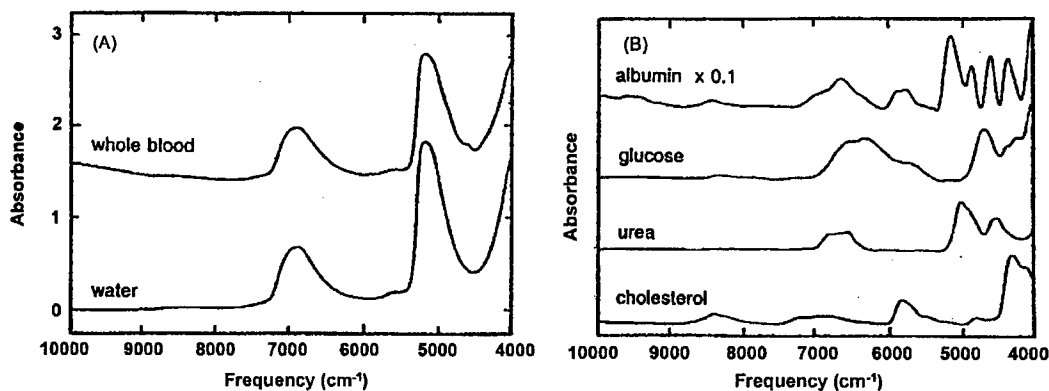


FIG. 10. NIR absorbance of interfering substances for glucose measurements. A: Whole blood and water. B: Albumin, urea, and cholesterol in comparison with glucose. Redrawn with permission from Heise.¹²

a new method is based on the assumption that glucose concentrations in tissue have a quantitative effect on the cutaneous microcirculation.⁴⁵ Controlled, periodic temperature variation enhances this effect and induces periodic vasoconstriction and vasodilatation, together with concomitant changes in light scattering by the skin. The reflectance signal is collected from different tissue depths and is then correlated with glucose values. Based on a calibration via an OGTT, glucose concentrations were predicted in three volunteers following the ingestion of different test meals. While the prediction was good for another OGTT, unconvincing data were obtained following high protein or low carbohydrate ingestion. Considerable improvement is necessary, therefore, to properly validate this approach for routine use.

Thermal emission spectroscopy correlates MIR emission, which depends on absolute body temperature and tissue composition, with blood glucose levels.⁴⁶ Enhanced sensitivity for glucose detection is theoretically possible as the information from the fingerprint region is used. In 20 subjects, thermal emission from the tympanic membrane allowed development of a calibration model, which was then used to predict serum glucose levels in six further volunteers. Good correlation ($r = 0.87$) was found, with all data falling in zones A and B of a Clarke Error Grid.

An alternative approach⁴⁷ is also based on "black body radiation," which changes when a temperature gradient is established across the tissue. Emission features at two different wavelengths—one representing a maximum for glucose absorption, the other a minimum—are recorded at different temperatures; a phase shift between the two signals is produced that is proportional to the increase in glucose concentration in the tissue. To date, however, the method has only been tested on tissue phantoms.

Raman spectroscopy

Raman spectroscopy is a powerful tool for studying a variety of biological molecules and tissues and is complementary to infrared.^{119,120} Logically, it is also being considered as a potential noninvasive glucose monitoring ap-

proach.^{7,8,121-123} Raman scattering is observed when monochromatic radiation is incident upon optically transparent media. As well as the transmitted/absorbed radiation, a portion of the light is scattered. Most of the latter is elastically scattered at the same wavelength. However, some of the incident light of frequency $\nu_0 = c/\lambda_0$ is inelastically scattered at frequencies equal to $\nu_0 \pm \nu_i$. The loss (Stokes shift) or gain (anti-Stokes shift) of photon energy is due to transitions between the rotational and/or vibrational energy states within the scattering molecule. In most cases, Raman bands of interest are shifted to longer wavelengths relative to the excitation wavelength. The observed shifts ν_i are independent of the excitation frequency ν_0 and are very characteristic of the scattering molecule. Like infrared, therefore, Raman spectra exhibit highly specific bands, which can be used to identify and quantify a molecule. Figure 11 shows a Raman spectrum for glucose in water. Because of anharmonic oscillations of dipoles (such as those for water), overtone frequencies are apparent as well as fundamental vibrations. In contrast to the infrared, though, the Raman overtones are much weaker. Raman spectra are therefore much simpler, and water causes much less interference. The Raman signal itself is nevertheless weak, and only recently have highly sensitive detectors been developed for the quantitative measurement of physiological glucose levels by Raman spectroscopy.⁷

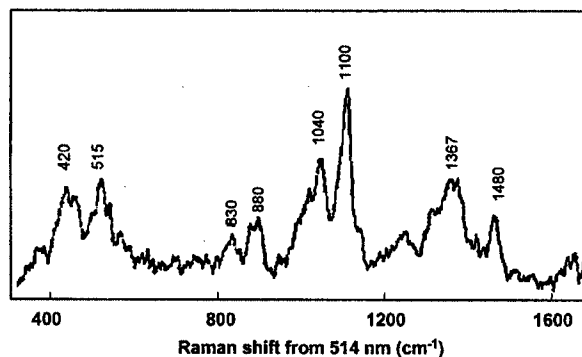


FIG. 11. Typical Raman spectrum for glucose in aqueous solution. The Raman spectrum is shown as vibrational intensity versus shift in wavenumbers from the 514 nm excitation wavelength. The background signal from water has been subtracted. Redrawn with permission from McNichols and Côté.⁷

F12

Because of the high background fluorescence in the skin and underlying tissues (from proteins and other molecules), the eye has been suggested as site for glucose measurements by Raman spectroscopy (Fig. 12).¹²²⁻¹²⁴ As for infrared methodologies, the use of multivariate statistical techniques and advances in instrumentation have increased significantly the feasibility of this idea.^{122,125,126} The primary drawback concerns the laser power necessary.⁸ To prevent injury, the power must be low, but this decreases the signal-to-noise ratio. While the high background fluorescence can be circumvented by the use of longer excitation wavelengths, the intensity of the Raman signal falls off with decreasing frequency as a function of ν_0^4 . Therefore, the optimal wavelength is a compromise between minimizing fluorescence and maintaining the Raman signal. In addition, for the precise quantification of glucose levels in vivo, the presence of other compounds that have overlapping Raman spectra must be taken into consideration (similar to infrared). Recent work using a confocal Raman microscope on an in vitro model for the anterior chamber of the eye has shown that glucose can be determined with clinically acceptable precision in the presence of the principal interfering substances, namely, urea, ascorbate, and lactate.¹²² However, safety and clinical efficacy remain to be assessed in vivo. Lastly, increased sensitivity for glucose measurements can be achieved

with surface-enhanced Raman scattering.¹²⁷ This approach, however, is invasive and would require implantation of a glucose binding sensor in the eye or subcutaneous tissue.

Polarimetry

Polarimetric quantification of glucose is based on the phenomenon of optical rotatory dispersion, whereby a chiral molecule in aqueous solution rotates the plane of linearly polarized light. This rotation is due to a difference in the indices of refraction (n_L and n_R) for, respectively, left and right circularly polarized light passing through a solution of the molecule in question.

The net rotation is expressed as:

$$\Phi = \alpha_\lambda \times l \times c \quad (3)$$

where α_λ is the specific rotation in degrees $\text{dm}^{-1} (\text{g/mL})^{-1}$ at wavelength λ , l is the pathlength (in dm), and c is the concentration (in g/L). Glucose in the body is dextrorotatory and has a specific rotation of $+52.6^\circ \text{ dm}^{-1} (\text{g/mL})^{-1}$ at the sodium D-line of 589 nm. Besides the parameters given in the equation, the rotation depends on the temperature and the pH of the solvent.

For polarimetry to be used for noninvasive glucose measurements, the signal must be able to pass from the source, through the body, and then to a detector without total depolarization of the beam. But, maintaining polarization information as the beam passes through tissue that may be as thick as 1 cm, and which includes skin, is not feasible. The polarimetric device would have to measure millidegree rotations under conditions of $>95\%$ depolarization of the light due to scattering. At the very least, this criterion demands a tissue thickness less than 4 mm, rendering the polarimetric measurement of glucose originating from the ISF unrealistic.¹²⁸

Alternatively, the polarimetric measurement of glucose in the eye is reasonable.^{7,129-132} For a normal glucose level of 100 mg/dL (5.55 mM), an optical rotation of ~ 4.562 millidegrees per optical pass would be observed, given a specific rotation of glucose of $45.62^\circ \text{ dm}^{-1} (\text{g/mL})^{-1}$ at 633 nm and a pathlength of 1 cm.

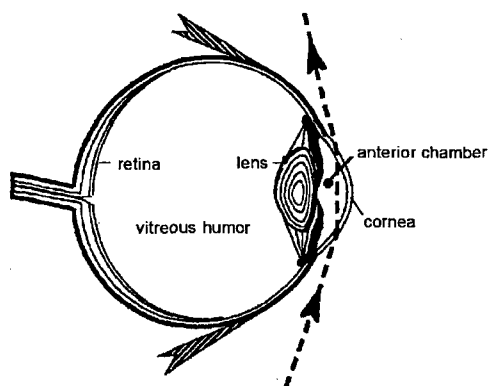


FIG. 12. Optical sensing in the anterior chamber of the eye. A typically proposed light path is shown, whereby incident light passes through and interacts with the aqueous humour. Redrawn with permission from McNichols and Coté.⁷

The latter corresponds to the approximate width of the anterior chamber of the eye (Fig. 12). Before this approach can be considered viable for glucose monitoring, however, the confounding rotation due to corneal birefringence and the variation in this rotation because of artifacts due to eye motion must be addressed.⁸ Recent work is attempting to systematically resolve this challenge via a better characterization of the problem and the development of new configurations.¹³³⁻¹³⁶ And, once more, the lag time between glucose concentrations in the blood and those in the aqueous humor needs to be assessed if accurate measurements of glycaemia are to be made in the eye.¹³⁷

Scattering

As described previously, light scattering is the other major optical interaction with tissue. In the NIR, scattering dominates over absorption.^{138,139} The scattering properties of a tissue depend on the relative refractive indices ("mismatch") of the scattering particle (n_2) and the medium (n_1) (Fig. 13A). If n_2 is greater than n_1 ,

significant scattering occurs; as the ratio n_2/n_1 falls, scattering decreases, and when n_2 equals n_1 , the scattering suspension is transparent. In vitro studies of turbid suspensions containing different levels of glucose have confirmed that increasing the glucose concentration results in decreased scattering.^{138,139} In vivo, as the glucose level is raised, the refractive indices of blood and ISF increase, while the refractive index of the scattering particles in the skin remains unchanged (Fig. 13B). Thus, the mismatch of the refractive indices is reduced, and scattering decreases. The opposite is observed when glucose levels fall.^{140,141}

Proof-of-concept for this approach has been demonstrated in healthy subjects and patients with diabetes, using a custom-built optical system operating at four different wavelengths.^{51,52} The decrease in the scattering coefficient was approximately 1.0% per 5.5 mM increase in blood glucose concentration and was relatively independent of the wavelength studied. However, considerable inter-individual differences in the scattering coefficient were reported, and drifting sensor responses, varying up to 2% over a 4-h period, were observed. Following an OGTT, 73% of the predicted glucose values were acceptable (defined as $r > 0.75$), and no differences between patients with diabetes and healthy individuals were observed. Sources of error included changes in the skin-sensor interface as a function of time, motion artifacts, temperature, and other physiological factors that altered the scattering properties in a manner independent of glucose levels. A 1 mM increase in glucose concentration was calculated to be equivalent to a 0.2% increase in water content, a 0.1% increase in protein level, or a 0.2% decrease in temperature.^{13,51} It follows that careful calibration is essential to avoid artifactual results. Nevertheless, the development of a small portable device makes this approach promising, and further in vivo data on a larger study population can be expected.

Optical coherence tomography (OCT)

A more sophisticated scattering technique is OCT. The method was first introduced to perform tomographic imaging of the eye¹⁴² and is

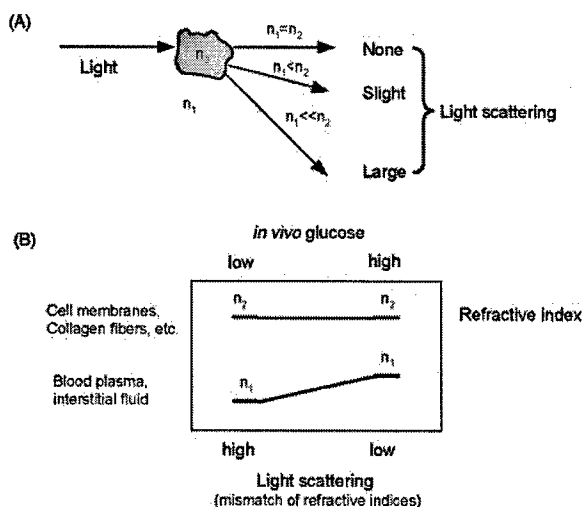


FIG. 13. Glucose measurements by light scattering. A: Scattering in turbid media depends on the ratio of refractive indices of the solvent (n_1) and the scattering particles (n_2). Scattering increases with a rising mismatch of refractive indices. B: An increase in glucose concentration in vivo increases the refractive index of blood and ISF (n_1), while the refractive index of the scattering particles in the skin (n_2) is not affected. Because of the lower mismatch of refractive indices, light scattering is therefore decreased. Redrawn with permission from Heinemann and Schmelzein-Redeker.⁵¹

now being actively developed for many diagnostic applications. An OCT system is a Michelson interferometer comprising a low coherent laser source, an in-depth scanning system, a sampling device, and a photodiode. After the skin is irradiated with coherent light, the backscattered radiation is recorded. Scattered, coherent light (with the same phase but a different optical pathlength) interferes with light from the incident source and provides information on the probed tissue depth. This allows two-dimensional examination of the tissue to a depth of 1 mm and lateral scans with high resolution (about 10 μm or less).¹⁴³

For the determination of glucose levels, the analysis is focused on the dermis, where a change in the OCT signal indicates a modified scattering coefficient and, hence, a different analyte concentration. The method was tested first in phantoms and in animals,¹⁴⁴ and then in healthy volunteers during an OGTT.¹⁴⁵ Good correlation ($0.8 \leq r \leq 0.95$) between blood glucose and the OCT signal was found: A difference of 10 mg/dL (0.56 mM) glucose corresponded to an average change of 1.9% in the OCT signal. Error sources are the same as those seen in all scattering approaches, including, for example, motion artifacts and tissue heterogeneity. Animal models have pointed out, in particular, the impact of motion artifacts and the presence of interfering species.¹⁴³ However, changes in the levels of urea, KCl, and NaCl, as well as moderate temperature ($\pm 1^\circ\text{C}$), blood pressure, heart rate, and hematocrit fluctuations, did not significantly alter the glucose signal. This method has potential, therefore, for accurate glucose monitoring and has the advantage of operating at a laser power that is safe. For glucose monitoring applications, studies in large populations and miniaturization of the apparatus without loss of sensitivity are now required.

Occlusion spectroscopy

Occlusion spectroscopy is a scattering technique that exploits the pulsatile nature of blood circulation.^{53,54} Because of time-dependent erythrocyte aggregation, the average size of scattering particles in the blood increases, and this results in decreased scattering and higher

transmission of light. For quantitative glucose measurements, the scattering signal is enhanced by applying an over-systolic pressure ("occlusion") on the finger of the patient for intervals of 2–3 s with intermittent pressure release. Artificial erythrocyte aggregation is thus induced, and the scattering signal is measured. In analogy to the extravascular response, the change in the scattering coefficient in blood depends on the mismatch between the erythrocyte and plasma refraction indices, with the latter being related to the blood glucose level. The measured differences are wavelength dependent, and scans over the frequency range allow the glucose-related signal to be extracted. With this method, hematocrit and oxygen content of blood can also be measured.⁵⁴ Glucose monitoring has been demonstrated on five subjects with diabetes.⁵³

Occlusion spectroscopy has the advantage of measuring glucose in the arterial component of vasculature, thus avoiding important lag times and complex glucose blood-ISF kinetics. Before the method can be widely applied, however, the calibration model needs to be improved, and possible interferences either from drugs influencing aggregation (anticoagulants, antiplatelet drugs) or postprandially from increased serum levels of nutrients (free fatty acids, chylomicrons) need to be evaluated.

Photoacoustic spectroscopy

The photoacoustic effect involves the creation of ultrasonic waves by the absorption of light. The tissue is illuminated with laser pulses at selected wavelengths. Part of the light energy is absorbed and can be dissipated by radiative processes (re-emission) or by non-radiative processes in which the absorbed energy causes localized heating of the sample. This produces a small temperature rise, which depends on the specific heat capacity, and leads in turn to a volumetric expansion of the region interacting with the light. Thereby, an ultrasonic pressure pulse is generated, which can be measured by a piezoelectric detector.¹⁴⁶

The application of the technique for glucose monitoring is based on the fact that with increasing glucose levels, the specific heat capacity of a solution decreases, while the

acoustic velocity of the pulse increases, thus allowing for quantification of the analyte. The use of MIR radiation to excite the sample suffers from the same disadvantages as classical infrared spectroscopy. More recently, therefore, NIR wavelengths have been used. After initial tests in aqueous solutions, gelatin-based tissue phantoms, and whole blood samples,^{13,146} preliminary *in vivo* studies in human volunteers were performed.^{147,148} Predicted blood glucose values, based on individual calibration, showed good precision, but substantial baseline shifts were also observed.¹⁴⁸

Further *in vitro* experiments investigated the effect of other blood analytes on the photoacoustic effect.¹⁴⁶ Spectra of sodium chloride, cholesterol, and bovine serum albumin were compared with that of glucose, and each showed a distinct photoacoustic response after subtraction of water. Interferences with other tissue components, however, remain to be studied, and it is anticipated that more sophisticated methods of data analysis (chemometrics), in combination with other optical methods, may be necessary for the determination of absolute blood glucose levels.

The photoacoustic approach has been developed to the level of a prototype wristwatch device.^{55,56} Initial studies on 10 healthy subjects and seven patients with diabetes showed good glucose prediction, with all data points falling in regions A and B of a Clarke Error Grid ($r =$

0.71). Photoacoustic measurements have a sensitivity superior to those of other NIR detection methods,¹³ and recent work underlines that the photoacoustic effect is coupled to the modified scattering coefficient in blood.^{149–151} Additional research is needed in identifying interfering sources and optimizing calibration.⁵⁷ Clinical data on a larger population with the prototype device (and more information about the apparatus) are now awaited.

IMPEDANCE SPECTROSCOPY

In contrast to the optical methods discussed so far, impedance spectroscopy (also referred to as radio-wave or dielectric spectroscopy) measures the dielectric properties of the tissue. A small alternating current is applied, and the impedance of the tissue to the current flow is recorded as a function of frequency. In skin research, impedance spectroscopy is a useful, noninvasive tool to assess transport properties and hydration of the tissue.¹⁵² The electric properties of the skin can be divided into a resistive component, which is attributed to skin appendages ("shunts"), and a capacitive component, corresponding to the lipid-protein matrix of the stratum corneum. Impedance spectroscopy over the 1 Hz–1 kHz range allows these two contributions to be separated. Skin impedance is sensitive to changes in membrane

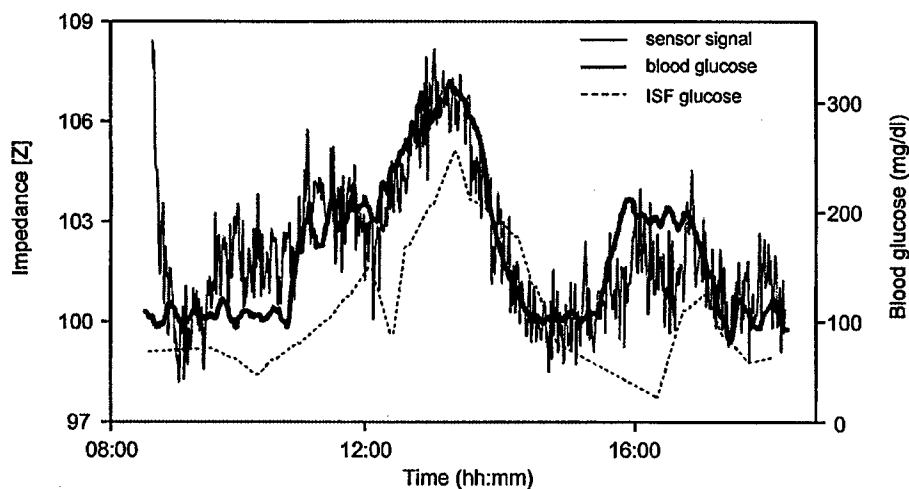


FIG. 14. Impedance signal measured at the skin surface compared with blood and ISF glucose concentrations in one subject. Redrawn with permission from Caduff et al.²⁹

potentials and, hence, to the movement of ions in the tissue.

At higher frequencies, between 1 and 200 MHz, the interfacial membrane polarization of erythrocytes and other cell membranes (the so-called Maxwell-Wagner effect) in the dermis can be studied. For glucose monitoring, the concentration-dependent interaction of glucose with the erythrocytes is measured, and results in a modified membrane potential.²⁸

An impedance sensor has been incorporated into a wristwatch device, and voltage measurements over the indicated frequency range allow the blood glucose concentration to be predicted with a mean impedance change of 0.5–0.8 Ω per 1 mM glucose.²⁹ Glucose clamp experiments and OGTT showed “good” correlation in five of eight human volunteers. Simultaneous microdialysis measurements confirmed that the glucose-related signal is recorded from blood rather than from the interstitial compartment (Fig. 14). Error sources include temperature, sweating, and motion artifacts. The influence of the latter on microvascular blood flow variations has been recently addressed.³⁰ Individual calibration is essential and requires sophisticated data acquisition over a 2-day period. More extended clinical data in patients with diabetes (for whom the electrolyte balance may be different and interfere with the measurement) are awaited.

F14

CONCLUSIONS

Noninvasive glucose monitoring is a broad and fast-moving field of research. In the past 5 years, several techniques have made significant progress or have been newly developed, and some have even become available to patients. From transdermal fluid extraction approaches, glucose-specific information is more easily obtained. However, some of these methods must still be refined to exclude aggressive extraction procedures, and must prove safety and performance on a larger scale. In the optical field, classical absorption-based methods are still hampered of a lack of sensitivity, and a number of techniques have evolved as a result to exploit other tissue properties such as light

scattering. For these devices, the development of appropriate calibration models separating the often weak glucose signal from the complex biological matrix represented by the skin is a real challenge. It remains difficult to predict, therefore, exactly when a portable and affordable optical device will eventually provide sensitive and accurate glucose measurements.

Although a remarkable technical achievement, the GlucoWatch (like all the technologies described here) requires calibration to a blood sample on at least a daily basis. For some approaches, the calibration procedure is even more intensive. Clearly, the goal of this field is a totally no-invasive technique that can make accurate measurements of glycaemia in a compartment that is relevant for subsequent diabetes therapy. The ultimate “Holy Grail,” of course, is an integrated device that uses the biofeedback from glucose monitoring to determine insulin input dose and rate. The need for such a system is clear in an era when the number of individuals with diabetes worldwide is continuing to grow at an alarming level.

ACKNOWLEDGMENTS

This work was supported by grant EB 001420 from the U.S. National Institutes of Health and grant DAMD17-02-1-0712 from the U.S. Army Medical Research Acquisition Activity.

REFERENCES

1. Jones M, Harrison JM: The future of diabetes technologies and therapeutics. *Diabetes Technol Ther* 2002;4:351–359.
2. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993;329:977–986.
3. UK Prospective Diabetes Study Group: Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 1998;352:837–853.
4. Ohkubo Y, Kishikawa H, Araki E, Miyata T, Isami S, Motoyoshi S, Kojima Y, Furuyoshi N, Shichizi M: Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese

- patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study. *Diabetes Res Clin Pract* 1995;28:103-117.
5. Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications Trial (EDIC) Research Group: Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy. *JAMA* 2003;290:2159-2167.
 6. Koschinsky T, Heinemann L: Sensors for glucose monitoring: technical and clinical aspects. *Diabetes Metab Res Rev* 2001;17:113-123.
 7. McNichols RJ, Coté GL: Optical glucose sensing in biological fluids: an overview. *J Biomed Opt* 2000;5:1-16.
 8. Coté GL: Noninvasive and minimally-invasive optical monitoring technologies for monitoring health and nutritional status in mothers and young children. *J Nutr* 2001;131:1596S-1604S.
 9. Klonoff DC: Noninvasive blood glucose monitoring. *Diabetes Care* 1997;20:433-437.
 10. Roe JN, Smoller BR: Bloodless glucose measurements. *Crit Rev Ther Drug Carr Syst* 1998;15:199-241.
 11. Pickup J, McCartney L, Rolinski O, Birch D: In vivo glucose sensing for diabetes management: progress towards non-invasive monitoring. *BMJ* 1999;319:1289-1301.
 12. Heise HM: Non-invasive monitoring of metabolites using near infrared spectroscopy: state of the art. *Horm Metab Res* 1996;28:527-534.
 13. Khalil OS: Spectroscopic and clinical aspects of non-invasive glucose measurements. *Clin Chem* 1999;45:165-177.
 14. Kerner W: Implantable glucose sensors: present status and future developments. *Exp Clin Endocrinol Diabetes* 2001;109(Suppl 2):S341-S346.
 15. McShane MJ: Potential for glucose monitoring with nanoengineered fluorescent biosensors. *Diabetes Technol Ther* 2002;4:533-538.
 16. Badugu R, Lakowicz JR, Geddes CD: A glucose sensing contact lens: a non-invasive technique for continuous physiological glucose monitoring. *J Fluoresc* 2003;13:371-374.
 17. Rao G, Guy RH, Glikfeld P, LaCourse WR, Leung L, Tamada JA, Potts RO, Azimi NT: Reverse iontophoresis: non invasive glucose monitoring in vivo in humans. *Pharm Res* 1995;12:1869-1873.
 18. Tamada JA, Bohannon NJV, Potts RO: Measurement of glucose in diabetics subjects using non invasive transdermal extraction. *Nat Med* 1995;1:1198-1201.
 19. Eastman RC, Chase HP, Buckingham B, Hathout EH, Fuller-Byk L, Leptien A, Van Wyhe MM, Davis TL, Fermi SJ, Pechler H, Sahyun G, Lopatin M, Wang BY, Wei C, Bartkowiak M, Ginsberg BH, Tamada JA, Pitzer KR: Use of the GlucoWatch Biographer in children and adolescents with diabetes. *Pediatr Diabetes* 2002;3:127-134.
 20. Potts RO, Tamada JA, Tierney MJ: Glucose monitoring by reverse iontophoresis. *Diabetes Metab Res Rev* 2002;18(Suppl):S49-S53.
 21. Kost J, Mitragotri S, Gabbay RA, Pishko M, Langer R: Transdermal monitoring of glucose and other analytes using ultrasound. *Nat Med* 2000;6:347-350.
 22. Mitragotri S, Coleman M, Kost J, Langer R: Analysis of ultrasonically extracted interstitial fluid as a predictor of blood glucose levels. *J Appl Physiol* 2000;89:961-966.
 23. Smart WH, Subramanian K, Orloff E, inventors: Silicon microprobe with integrated biosensor. US patent application 20020137998. September 26, 2002.
 24. Smart WH, Subramanian K: The use of silicon microfabrication technology in painless blood glucose monitoring. *Diabetes Technol Ther* 2000;2:549-559.
 25. Gebhardt S, Faupel M, Fowler R, Kapsner C, Lincoln D, McGee V, Pasqua J, Steed L, Wangsness M, Xu F, Vanstorp M: Glucose sensing in transdermal body fluid collected under continuous vacuum pressure via micropores in the stratum corneum. *Diabetes Technol Ther* 2003;5:159-166.
 26. Stout PJ, Peled N, Erckson BJ, Hilgers ME, Racchini JR, Hoegh TB: Comparison of glucose levels in dermal interstitial fluid and finger capillary blood. *Diabetes Technol Ther* 2001;3:81-90.
 27. Collison ME, Stout PJ, Glushko TS, Pokela KN, Mullins-Hirte DJ, Racchini JR, Walter MA, Mecca SP, Rundquist J, Allen JJ, Hilgers ME, Hoegh TB: Analytical characterization of electrochemical biosensor test strips for measurement of glucose in low-volume interstitial fluid samples. *Clin Chem* 1999;45:1665-1673.
 28. Hayashi Y, Livshits L, Caduff A, Feldman Yu: Dielectric spectroscopy study of specific glucose influence on human erythrocyte membranes. *J Phys D Appl Phys* 2003;36:369-374.
 29. Caduff A, Hirt E, Feldman Yu, Ali Z, Heinemann L: First human experiments with a novel non-invasive, non-optical continuous glucose monitoring system. *Biosens Bioelectron* 2003;19:209-217.
 30. Pfützner A, Caduff A, Larbig M, Schrepfer T, Forst T: Impact of posture and fixation technique on impedance spectroscopy used for continuous and non-invasive glucose monitoring. *Diabetes Technol Ther* 2004;6:435-441.
 31. Malin SF, Ruchti TL, Blank TB, Thennadil SN, Monfre SL: Noninvasive prediction of glucose by near-infrared diffuse reflectance spectroscopy. *Clin Chem* 1999;45:1651-1658.
 32. Blank TB, Ruchti TL, Lorenz AD, Monfre SL, Makarewicz MR, Mattu M, Hazen KH: Clinical results from a non-invasive blood glucose monitor. *Proc SPIE* 2002;4624:1-10.
 33. Ruchti TL, Blank TB, Lorenz AD, Makarewicz MR, Mattu M: Evaluation of a non-invasive glucose sensor [abstract]. *Diabetes Technol Ther* 2004;6:269.
 34. Fleming CM, Davies HT, Ratner R, Brown CW, Hull EL, Maynard JD: Use of NIR spectroscopy to detect diabetes [abstract]. *Diabetes Technol Ther* 2004;6:243.
 35. Robinson MR, Fleming C, Jones H, Rohrscheib M, inventors; InLight Solutions, Inc., assignee: Appara-

- tus and method for non-invasive spectroscopic measurement of analytes in tissue using a matched reference analyte. US patent application 20030191377. October 9, 2003.
36. Harjunmaa H, Kun S, Burrell R, Leuenberger J, Lock JP, Peura RA: Ex-vivo measurements of a non-invasive continuous blood glucose monitor [abstract]. *Diabetes Technol Ther* 2004;6:249.
 37. Harjunmaa H, Kun S, Peura RA, Rolls JA, inventors: Non-invasive substance concentration measurement using and [sic] optical bridge. US patent application 20030204133. October 30, 2003.
 38. Harjunmaa H, Mendelson Y, Wang Y, inventors; Vivascan Corporation, assignee: Electromagnetic method and apparatus to measure constituents of human or animal tissue. US patent 5,178,142. January 12, 1993.
 39. Maruo K, Tsurugi M, Tamura M, Ozaki Y: In vivo noninvasive measurement of blood glucose by near-infrared diffuse-reflectance spectroscopy. *Appl Spectrosc* 2003;57:1236-1244.
 40. Maruo K, Tsurugi M, Chin J, Ota T, Arimoto H, Yamada Y, Tamura M, Ishii M, Ozaki Y: Noninvasive blood glucose assay using a newly developed near-infrared system. *IEEE J Sel Top Quant Electron* 2003;9:322-330.
 41. Pawluczyk R, Scieczna T, Cadell TE: Method for determination of analytes using near infrared, adjacent visible spectrum and an array of longer near infrared wavelengths. CA patent application 2383727. August 31, 2000.
 42. Samsoodar J, Ashwani K, Geng L, Lynch M: Non-invasive measurement of HbA1c using near infrared spectroscopy [abstract]. *Diabetes Technol Ther* 2003;5:269.
 43. Sodickson LA, Block MJ: Kromoscopic analysis: a possible alternative to spectroscopic analysis for noninvasive measurement of analytes in vivo. *Clin Chem* 1994;40:1838-1844.
 44. Block MJ, Misner MW, Guthermann HE: Comparative in vitro and non-invasive in vivo Kromoscopic measurements. *J Near Infrared Spectrosc* 2002;10:165-176.
 45. Yeh SJ, Hanna CF, Khalil OS: Monitoring blood glucose changes in cutaneous tissue by temperature-modulated localized reflectance measurements. *Clin Chem* 2003;49:924-934.
 46. Malchoff CD, Shoukri K, Landau JI, Buchert JM: A novel noninvasive blood glucose monitor. *Diabetes Care* 2002;25:2268-2275.
 47. Zheng P, Kramer CE, Barnes CW, Braig JR, Sterling BB: Noninvasive glucose determination by oscillating thermal gradient spectrometry. *Diabetes Technol Ther* 2000;2:17-25.
 48. Berman HL, Roe JN, inventors: Infrared ATR glucose measurement system (II). US patent application 20020151773. October 17, 2002.
 49. Chaiken J, Peterson CM, inventors; LighTouch Medical, Inc., assignee: Method for non-invasive measurement of an analyte. US patent 6,377,828. April 23, 2002.
 50. Chaiken J, Peterson CM; LighTouch Medical, Inc., assignee: Method for non-invasive measurement of an analyte. US patent 6,044,285. March 28, 2000.
 51. Heinemann L, Schmelzeisen-Redeker G: Non-invasive continuous glucose monitoring in Type I diabetic patients with optical glucose sensors. *Diabetologia* 1998;41:848-854.
 52. Heinemann L, Krämer U, Klötzer HM, Hein M, Volz D, Hermann M, Heise T, Rave K: Noninvasive glucose measurement by monitoring of scattering coefficient during oral glucose tolerance tests. *Diabetes Technol Ther* 2000;2:211-220.
 53. Cohen O, Fine I, Monashkin E, Karasik A: Glucose correlation with light scattering patterns—a novel method for non-invasive glucose measurements. *Diabetes Technol Ther* 2003;5:11-17.
 54. Fine I, Fikhte B, Shvartsman LD: Occlusion spectroscopy as a new paradigm for noninvasive blood measurements. *Proc SPIE* 2001;4263:122-130.
 55. Pesach B, Nagar R, Belfer I, Argaman D, Askenazi S, Adoram A, Roffeh Y: Continuous non-invasive venous blood glucose monitor [abstract]. *Diabetes Technol Ther* 2003;5:264.
 56. Raz I, Wainstein J, Argaman D: Continuous non-invasive venous blood glucose monitor [abstract]. *Diabetes* 2003;52(Suppl 1):A98.
 57. Grinberg L, Ben-David M, Antebi A, Rahimi H, Bitton G: Ex-vivo measurements of non-invasive continuous blood glucose monitor [abstract]. *Diabetes Technol Ther* 2004;6:248.
 58. Yum SL, Roe J: Capillary blood sampling for self-monitoring of blood glucose. *Diabetes Technol Ther* 1999;1:29-37.
 59. Pellet MA, Hadgraft J, Roberts MS: The back diffusion of glucose across human skin in vitro. *Int J Pharm* 1999;193:27-35.
 60. Freinkel RK: Carbohydrate metabolism of epidermis. In: Goldsmith LA, ed. *Physiology, Biochemistry, and Molecular Biology of the Skin*. New York: Oxford University Press, 1993:452-461.
 61. Halprin KM, Ohkawara A: Glucose utilization in the human epidermis; its control by hexokinase. *J Invest Dermatol* 1966;46:278-282.
 62. Schurer NY, Elias PM: The biochemistry and function of stratum corneum lipids. *Adv Lipid Res* 1991;24:27-56.
 63. Cunningham DD, Young DF: Measurement of glucose on the skin surface, in stratum corneum and in transcutaneous extracts: implications for physiological sampling. *Clin Chem Lab Med* 2003;41:1224-1228.
 64. Cheong WF, Prael SA, Welch AL: A review of the optical properties of biological tissues. *IEEE J Quant Electron* 1990;26:2166-2185.
 65. van Gemert MJC, Jacques SL, Sterenborg HJCM, Star WM: Skin optics. *IEEE Trans Biomed Eng* 1989;36:1146-1154.

66. Anderson RR: Optics of the skin. In: Lim HW, Soter NA, eds. *Clinical Photomedicine*. New York: Marcel Dekker, 1993:19-35.
67. Burmeister JJ, Arnold MA: Evaluation of measurement sites for noninvasive blood glucose sensing with near-infrared transmission spectroscopy. *Clin Chem* 1999;45:1621-1627.
68. Aussedat B, Dupire-Angel M, Gifford R, Klein JC, Wilson GS, Reach G: Interstitial glucose concentration and glycemia: implications for continuous subcutaneous glucose monitoring. *Am J Physiol Endocrinol Metab* 2000;278:E716-E728.
69. Rebrin K, Steil GM, Van Antwerp WP, Mastrototaro JJ: Subcutaneous glucose predicts plasma glucose independent of insulin: implications for continuous monitoring. *Am J Physiol Endocrinol Metab* 1999;277:E561-E571.
70. Rebrin K, Steil GM: Can interstitial glucose assessment replace blood glucose measurements? *Diabetes Technol Ther* 2000;2:461-472.
71. Kulcu E, Tamada JA, Reach G, Potts RO, Lesho MJ: Physiological differences between interstitial glucose and blood glucose measured in human subjects. *Diabetes Care* 2003;26:2405-2409.
72. Regittnig W, Ellmerer M, Fauler G, Sendlhofer G, Trajanoski Z, Leis HJ, Schaupp L, Wach P, Pieber TR: Assessment of transcapillary glucose exchange in human skeletal muscle and adipose tissue. *Am J Physiol Endocrinol Metab* 2003;285:E241-E251.
73. Sternberg F, Meyerhoff C, Mennel FJ, Mayer H, Bischof F, Pfeiffer EF: Does fall in tissue glucose precede fall in blood glucose? *Diabetologia* 1996;39:609-612.
74. Moberg E, Hagstrom-Toft E, Arner P, Bolinder J: Protracted glucose fall in subcutaneous adipose tissue and skeletal muscle compared with blood during insulin-induced hypoglycemia. *Diabetologia* 1997;40:1320-1326.
75. Burnette RR: Iontophoresis. In: Hadgraft J, Guy RH, eds. *Transdermal Drug Delivery*. New York: Marcel Dekker, 1989:247-291.
76. Rao G, Glikfeld P, Guy RH: Reverse iontophoresis: development of a non invasive approach for glucose monitoring. *Pharm Res* 1993;10:1751-1755.
77. Tierney MJ, Tamada JA, Potts RO, Jovanovic L, Garg S; the Cygnus Research Team: Clinical evaluation of the GlucoWatch Biographer: a continual, non-invasive glucose monitor for patients with diabetes. *Biosens Bioelectron* 2001;16:621-629.
78. Tamada JA, Garg S, Jovanovic L, Pitzer KR, Fermi S, Potts RO: Noninvasive glucose monitoring—comprehensive clinical results. *JAMA* 1999;282:1839-1844.
79. Pitzer KR, Desai S, Dunn T, Edelmann S, Yakalakshmi Y, Kennedy J, Tamada JA, Potts RO: Detection of hypoglycemia with the Glucowatch Biographer. *Diabetes Care* 2001;24:881-885.
80. Garg SK, Potts RO, Ackerman NR, Fermi SJ, Tamada JA, Chase HP: Correlation of fingerstick blood glucose measurements with Glucowatch Biographer glucose results in young subjects with type 1 diabetes. *Diabetes Care* 1999;22:1708-1714.
81. Clarke WL, Cox D, Gonder-Frederick LA, Carter W, Pohl SL: Evaluating clinical accuracy of systems for self-monitoring of blood glucose. *Diabetes Care* 1987;10:622-628.
82. The Diabetes Research in Children Network (DirecNet) Study Group: Accuracy of the Glucowatch G2 Biographer and the continuous glucose monitoring system during hypoglycemia. *Diabetes Care* 2004;27:722-726.
83. Tamada JA, Lesho M, Desai S, Eastman R: Detection of and prediction algorithms for hypoglycemia [abstract]. *Diabetes Technol Ther* 2004;6:225.
84. Tsalikian E, Kollman C, Fiallo-Scharrer R, Beck RW, Buckingham BA, Mauras N, Weinzimer S, Tamborlane W, Ruedy K; the Diabetes Research in Children Network (DirecNet) Study Group: Down alert function of the Glucowatch® G2™ Biographer (GW2B) during insulin-induced hypoglycemia in children [abstract]. *Diabetes Technol Ther* 2004;6:276-277.
85. Sieg A, Guy RH, Delgado-Charro MB: Reverse iontophoresis for noninvasive glucose monitoring: the internal standard concept. *J Pharm Sci* 2003;92:2295-2302.
86. Sieg A, Guy RH, Delgado-Charro MB: Noninvasive glucose monitoring by reverse iontophoresis in vivo: application of the internal standard concept. *Clin Chem* 2004;50:1383-1390.
87. Sieg A, Guy RH, Delgado-Charro MB: Simultaneous extraction of urea and glucose by reverse iontophoresis in vivo. *Pharm Res* 2004;21:1805-1810.
88. Knoll M, Adam S, Bahr E, Eshold J, Ross B, Steinkuhl R, Sundermeier C, Haueter U, Reihl B, Vering T: Minimally invasive suction sampling unit for interstitial fluid enhanced by electroosmotic mass transport. *Sens Actuators B* 2002;87:150-158.
89. Kost J: Ultrasound-assisted insulin delivery and non-invasive glucose sensing. *Diabetes Technol Ther* 2002;4:489-497.
90. Mitragotri S, Kost J: Low-frequency sonophoresis. A review. *Adv Drug Deliv Rev* 2004;56:589-601.
91. Cantrell JT, McArthur MJ, Pishko M: Transdermal extraction of interstitial fluid by low-frequency ultrasound quantified with $^3\text{H}_2\text{O}$ as tracer molecule. *J Pharm Sci* 2000;89:1170-1179.
92. Mitragotri S, Coleman M, Kost J, Langer R: Transdermal extraction of analytes using low-frequency ultrasound. *Pharm Res* 2000;17:466-470.
93. Chuang H, Taylor E, Davidson TW: Clinical evaluation of a continuous minimally invasive glucose flux sensor placed over ultrasonically permeated skin. *Diabetes Technol Ther* 2004;6:21-30.
94. Tierney MJ, Kim HL, Burns MD, Tamada JA, Potts RO: Electroanalysis of glucose in transcutaneously extracted samples. *Electroanalysis* 2000;12:666-671.
95. Kiistala U: Suction blister device for separation of viable epidermis from dermis. *J Invest Dermatol* 1968;50:129-137.

96. Kutlu N, Svedman P: Suction blister wounding, morphological and functional aspects. *Vasc Surg* 1992; 26:388-399.
97. Svedman P, Svedman C: Skin mini-erosion sampling technique: feasibility study with regard to serial glucose measurement. *Pharm Res* 1998;15:883-888.
98. Groth S, Staberg B: Suction blisters of the skin: a compartment with physiological, interstitium-like properties. *Scand J Clin Lab Invest* 1984;44:311-316.
99. Volden G, Thorsrud AK, Bjornson I, Jellum E: Biochemical composition of suction blister fluid determined by high resolution multicomponent analysis (capillary gas chromatography-mass spectrometry and two-dimensional electrophoresis). *J Invest Dermatol* 1980;75:421-424.
100. Jensen BM, Bjerring P, Christiansen JS, Orskov H: Glucose content in human skin—relationship with blood-glucose levels. *Scand J Clin Lab Invest* 1995;55:427-432.
101. Svedman C, Samra JS, Clark ML, Levy JC, Frayn KN: Skin mini-erosion technique for monitoring metabolites in interstitial fluid: its feasibility demonstrated by OGTT results in diabetic and non-diabetic subjects. *Scand J Clin Lab Invest* 1999;59:115-124.
102. Paranjape M, Garra J, Brida S, Schneider T, White R, Currie J: A PDMS dermal patch for non-intrusive transdermal glucose sensing. *Sens Actuator A* 2003;104:195-204.
103. Prausnitz MR: Microneedles for transdermal drug delivery. *Adv Drug Deliv Rev* 2003;56:581-587.
104. Smart WH, Subramanian K: The use of silicon microfabrication technology in painless blood glucose monitoring. *Diabetes Technol Ther* 2000;2:549-559.
105. Wang PM, Cornwell MG, Prausnitz MR: Transdermal microinfusion and extraction using hollow microneedles [abstract 851]. In: *Proceedings of the 30th International Symposium on Controlled Release of Bioactive Materials [CD-ROM]*. Minneapolis, MN: Controlled Release Society, 2003.
106. Kayashima S, Arai T, Noritake M, Nagata N, Kikuchi K, Ito N, Matsumoto Y, Kaneyoshi A, Kimura J, Kuriyama T: New transcutaneous sampling of glucose for patients with type II diabetes using an ion-sensitive field-effect transistor. *Clin Chim Acta* 1995;240:11-19.
107. Kayashima S, Arai T, Kikuchi M, Nagata N, Ito N, Kuriyama T, Kimura J: Suction effusion fluid from skin and constituent analysis: new candidate for interstitial fluid. *Am J Physiol* 1992;263:H1623-H1627.
108. Ito N, Kayashima S, Kimura J, Kuriyama T, Arai T, Kikuchi M, Nagata N: Development of a transcutaneous blood-constituent monitoring method using a suction effusion fluid collection technique and an ion-sensitive field-effect transistor glucose sensor. *Med Biol Eng Comput* 1994;32:242-246.
109. Shen YC, Davies AG, Linfield EH, Taday PF, Arnone DD: The use of Fourier-transform infrared spectroscopy for the quantitative determination of glucose concentration in whole blood. *Phys Med Biol* 2003;48:2023-2032.
110. Gabriely L, Wozniak R, Mevorach M, Kaplan J, Aharon Y, Shamoon H: Transcutaneous glucose measurement using near-infrared spectroscopy during hypoglycemia. *Diabetes Care* 2003;22:2026-2032.
111. Arimoto H, Tarumi M, Yamada Y: Temperature-insensitive measurement of glucose concentration based on near infrared spectroscopy and partial least squares analysis. *Opt Rev* 2003;10:74-76.
112. Huang L, Ding HS, Wang GZ: The preliminary study on noninvasive detection using NIR diffusion reflectance spectrum for monitoring blood glucose. *Spectrosc Spectr Anal* 2002;22:387-391.
113. Helwig AM, Arnold MA, Small GW: Evaluation of kromoscopy: resolution of glucose and urea. *Appl Opt* 2000;39:4715-4720.
114. Misner MW, Block MJ: Noninvasive measurement by kromoscopic analysis. *Spectroscopy* 2000;15: 51-55.
115. Amerov AK, Arnold MA: In vitro kromoscopic analysis of glucose in blood. *Proc SPIE* 2003;4965:7-16.
116. Block MJ, Misner MW, Guthermann HE: Comparative in vitro and non-invasive in vivo kromoscopic measurements. *J Near Infrared Spectrosc* 2002;10: 165-176.
117. Guthermann HE, Misner MW, Block MJ: In vitro to in vivo calibration transfer in Kromoscopy [abstract]. In: *Abstracts of Papers of the American Chemical Society, Vol. 224*. Washington, DC: American Chemical Society, 2002:019-ANYL.
118. Amerov AK, Sun Y, Small GW, Arnold MA: Kromoscopic measurement of glucose in the first overtone region of the near infrared spectrum. *Proc SPIE* 2002;4624:11-19.
119. Lawson EE, Barry BW, Williams AC, Edwards HGM: Biomedical applications of Raman spectroscopy. *J Raman Spectrosc* 1997;28:111-117.
120. Schrader B, Dippel B, Fendel S, Keller S, Löchte T, Riedl M, Schulte R, Tatsch E: NIR FT Raman spectroscopy—a new tool in medical diagnosis. *J Mol Struct* 1997;408/409:23-31.
121. Berger AJ, Itzkan I, Feld MS: Feasibility of measuring blood glucose concentration by near infrared Raman spectroscopy. *Spectrochim Acta A* 1997;53: 287-292.
122. Lambert JL, Morookian JM, Sirk SJ, Borchert MS: Measurement of aqueous glucose in a model anterior chamber using Raman spectroscopy. *J Raman Spectrosc* 2002;33:524-529.
123. Wicksted JP, Erckens RJ, Motamedi M, March W: Monitoring of aqueous humour metabolites using Raman spectroscopy. *Proc SPIE* 1994;2135:264-274.
124. Wang SY, Hasty CE, Watson PA, Wicksted JP, Stith RD, March WF: Analysis of metabolites in aqueous solutions by using laser Raman spectroscopy. *Appl Opt* 1993;32:925-929.
125. Berger AJ, Koo TW, Itzkan I, Feld MS: A method of linear multivariate calibration. *Anal Chem* 1998;70: 623-627.
126. Spiegelman CH, McShane MJ, Coté GL, Goetz MJ, Motamedi M, Yue QL: Theoretical justification of

- wavelength selection in PLS calibration: development of a new algorithm. *Anal Chem* 1998;70:35-44.
127. Yonzon CR, Haynes CL, Zhang X, Walsh JT, Van Duyne RP: A glucose biosensor based on surface-enhanced Raman scattering: improved partition layer, temporal stability, reversibility, and resistance to serum protein interference. *Anal Chem* 2004;76:78-85.
128. Varadan VK, Whitchurch A, Saurkesi K: Non-invasive biosensor and wireless interrogating system for glucose in blood. *Proc SPIE* 2003;5055:140-146.
129. Cameron BD, Coté GL: Noninvasive glucose sensing utilizing a digital closed-loop polarimetric approach. *IEEE Trans Biomed Eng* 1997;44:1221-1227.
130. March W, Engerman R, Rabinovitch B: Optical monitor of glucose. *Trans ASAO* 1979;25:28-31.
131. March WF, Rabinovitch B, Adams RL: Noninvasive glucose monitoring of the aqueous humour of the eye: Part II. Animal studies and the scleral lens. *Diabetes Care* 1982;5:259-265.
132. King TW, Coté GL, McNichols R, Goetz MJJ: Multi-spectral polarimetric glucose detection using a single Pockels cell. *Opt Eng* 1994;33:2746-2753.
133. Baba JS, Cameron BD, Theru S, Coté GL: Effect of temperature, pH, and corneal birefringence on polarimetric glucose monitoring in the eye. *J Biomed Opt* 2002;7:321-328.
134. Baba JS, Cooper CT, Coté GL: Modelling the rabbit's eye with the Mueller matrix for birefringent properties. *Proc SPIE* 2003;4965:104-110.
135. Baba JS, Coté GL: Dual-detector polarimetry for compensation of motion artifact in a glucose sensing system. *Proc SPIE* 2002;4624:76-80.
136. Boeckle S, Rovati LL, Ansari RR: Polarimetric glucose sensing using Brewster-reflection applying a rotating retarder analyzer. *Proc SPIE* 2003;5143:114-125.
137. Cameron BD, Baba JS, Coté GL: Measurement of the glucose transport time delay between the blood and aqueous humor of the eye for the eventual development of a noninvasive glucose sensor. *Diabetes Technol Ther* 2001;3:201-207.
138. Kohl M, Cope M, Essenpreis M, Bocker D: Influence of glucose concentration on light scattering in tissue-simulating phantoms. *Opt Lett* 1994;19:2170-2172.
139. Kohl M, Essenpreis M, Cope M: The influence of glucose concentration upon the transport of light in tissue-simulating phantoms. *Phys Med Biol* 1995;40:1267-1287.
140. Bruulsema JT, Hayward JE, Farrell TJ, Patterson MS, Heinemann L, Berger M, Koschinsky T, Sandahl-Christiansen J, Orskov H: Correlation between blood glucose concentration in diabetics and noninvasively measured tissue optical scattering coefficient. *Opt Lett* 1997;22:190-192.
141. Maier JS, Walker SA, Fantini S, Franceschini MA, Gratton E: Possible correlation between blood glucose concentration and the reduced scattering coefficient of tissues in the near infrared. *Opt Lett* 1994;19:2062-2064.
142. Huang D, Swanson EA, Lin CP, Schuman JS, Stinson WG, Chang W, Hee MR, Flotte T, Gregory K, Puliafito CA, Fujimoto JG: Optical coherence tomography. *Science* 1991;254:1178-1181.
143. Larin KV, Motamedi M, Ashitkov TV, Esenaliev RO: Specificity of non-invasive blood glucose sensing using optical coherence tomography: a pilot study. *Phys Med Biol* 2003;48:1371-1390.
144. Esenaliev RO, Larin KV, Larina IV, Motamedi M: Noninvasive monitoring of glucose concentration with optical coherence tomography. *Opt Lett* 2001;26:992-994.
145. Larin KV, Eledrisi MS, Motamedi M, Esenaliev RO: Noninvasive blood glucose monitoring with optical coherence tomography: a pilot study in human subjects. *Diabetes Care* 2002;25:2263-2267.
146. MacKenzie HA, Ashton HS, Spiers S, Shen Y, Freeborn SS, Hannigan J, Lindberg J, Rae P: Advances in photoacoustic noninvasive glucose testing. *Clin Chem* 1999;45:1587-1595.
147. Spanner G, Niessner R: Noninvasive determination of blood constituents using an array of modulated laser diodes and a photoacoustic sensor head. *Fresenius J Anal Chem* 1996;355:327-328.
148. MacKenzie HA, Ashton HS, Shen YC, Lindberg J, Rae P, Quan KM, Spiers S: Blood glucose measurements by photoacoustics. *OSA TOPS* 1998;22:156-159.
149. Zhao Z, Myllylä RA: Photoacoustic determination of glucose concentration in whole blood by a near-infrared laser diode. *Proc SPIE* 2001;4256:77-83.
150. Zhao Z, Myllylä RA: Photoacoustic blood glucose and skin measurement based on optical scattering effect. *Proc SPIE* 2002;707:153-157.
151. Shen Y, Spiers S, MacKenzie HA, Hugh A: Time resolved aspects of pulsed photoacoustic spectroscopy. *Anal Sci* 2001;17:s221-s222.
152. Burnette RR, DeNuzzio JD: Impedance spectroscopy: applications to human skin. In: Potts RO, Guy RH, eds. *Mechanisms of Transdermal Drug Delivery*. New York: Marcel Dekker, 1997:215-230.

Address reprint requests to:

M. Begoña Delgado-Charro

Department of Pharmacy and Pharmacology

University of Bath

Claverton Down

Bath, BA2 7AY, UK

E-mail: B.Delgado-Charro@bath.ac.uk

Reverse Iontophoresis for Noninvasive Glucose Monitoring: The Internal Standard Concept

ANKE SIEG,^{1,2} RICHARD H. GUY,^{1,2} M. BEGOÑA DELGADO-CHARRO^{1,2}

¹School of Pharmacy, University of Geneva, Quai Ernest-Ansermet 30, 1211 Geneva 4, Switzerland

²Centre interuniversitaire de recherche et d'enseignement, "Pharmapeptides", Parc d'affaires international, 74160 Archamps, France

Received 26 February 2003; revised 6 May 2003; accepted 9 May 2003

ABSTRACT: Reverse iontophoresis is used by the GlucoWatch® Biographer to non-invasively extract glucose across the skin, allowing a diabetic's glycemia to be evaluated every 10 min over several hours. However, before each use, the device must be calibrated with a blood sample assayed in the conventional way. The objective of this study was to identify an approach by which to avoid this invasive step. The dermal (anodal) side of porcine skin *in vitro* was bathed in buffered (pH 7.4) solutions containing glucose, at concentrations from 3 to 10 mM, and physiological levels of sodium chloride. Constant current was applied and the cathodal solution contacting the outer skin surface was analyzed periodically for the quantities of Na⁺ and glucose extracted by "reverse" electromigration and electroosmosis, respectively. Although the extracted Na⁺ flux was invariant, as expected, given the essentially fixed NaCl concentration present in the physiological system, the glucose samples reflected proportionately the subdermal concentration. Equally, the extracted flux ratio (glucose/sodium) varied linearly with the subdermal glucose/sodium concentration ratio; knowing the gradient of this correlation, therefore, means that a measurement of the extraction flux ratio can be used to determine the subdermal glucose concentration (the physiological [Na⁺] being known and fixed). Thus, a refinement of the reverse iontophoresis technology using the simultaneous determination of the extracted fluxes of the analyte of interest (glucose) and of an "internal standard", whose level in the biological system is invariant (Na⁺), may permit a noninvasive sampling methodology free of the need for calibration with a blood sample. © 2003 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 92:2295–2302, 2003

Keywords: skin; transdermal; iontophoresis; noninvasive glucose monitoring; electroosmosis

INTRODUCTION

Frequent self-monitoring of glucose is essential for a safe and effective diabetes therapy. Intensified insulin treatment coupled to frequent monitoring significantly reduces the long-term complications

of diabetes mellitus,¹ but also increases the risk of hypoglycemic events. Conventional self-testing methods require a drop of blood for each glucose measurement, a painful and inconvenient procedure with poor patient compliance. Consequently, there has been, and continues to be, a considerable investment in the development of noninvasive and continuous glucose monitoring technologies.²

Iontophoresis is one such approach, which uses a small electric current to drive charged and highly polar compounds through the skin at rates very much greater than their passive permeabilities.

Correspondence to: Richard H. Guy (Telephone: +33 (0) 450 31 50 21; Fax: +33 (0) 450 95 28 32; E-mail: Richard.Guy@pharm.unige.ch)

Journal of Pharmaceutical Sciences, Vol. 92, 2295–2302 (2003)
© 2003 Wiley-Liss, Inc. and the American Pharmacists Association

Two major mechanisms are involved: electromigration and electroosmosis.³ Electromigration is the movement of small ions across the skin and a direct consequence of the electric field applied. Electron fluxes are transformed into ionic fluxes via the electrode reactions. Ionic transport proceeds as if through the skin to maintain electroneutrality.⁴ Electroosmosis is the principal transport mechanism of uncharged molecules and of high molecular weight cations. The skin is negatively charged at physiological pH, and is thus permselective to positive ions.⁵ This preferential passage of counterions induces an electroosmotic solvent flow that carries neutral molecules in the anode-to-cathode direction. The symmetry of iontophoresis renders it useful not only for drug delivery into the body, but also for the extraction of endogenous substances of clinical interest, and, in particular, glucose.⁶

This latter application has been previously investigated in depth, and has led to the development of the GlucoWatch® Biographer (Cygnus Inc., Redwood City, CA).⁷⁻¹¹ The wrist-worn device monitors glucose continuously for up to 13 h, recording six glucose readings per hour. However, before each use, it must be calibrated with a conventional blood sample to correlate the extracted glucose amounts with subdermal levels. This essential step has been perceived as a disadvantage despite the fact that the GlucoWatch provides tremendously more information to the diabetic than one or two "finger-sticks" per day (the current, normal situation). Right or wrong, the fact that this new advance does not eliminate the need for an invasively obtained blood sample has led some to ask "why should I invest in (admittedly) a more sophisticated device if I still have to prick my finger twice a day?" It follows that a completely non-invasive calibration approach would be beneficial and would open the way, perhaps, to other applications of the reverse iontophoresis technology.

The concept addressed here is that of an internal standard. As iontophoresis is nonspecific, many ions and small uncharged species (that is, in addition to the analyte of interest) are moved across the skin when the current is applied. Instead of detecting uniquely the single-target substance extracted by iontophoresis and calibrating its transdermal measurement via a blood assay, we propose to monitor the extraction of two species simultaneously: the compound of interest, the temporal change in whose concentration is of clinical importance (i.e., glucose), and a second analyte, the physiological concentration of which

is known and essentially fixed. If the iontophoretic transport of the analyte (A) and the latter "internal standard" (IS) are independent of one another, then their fluxes (J) into collection devices on the skin surface should obey the relationship:

$$J_A/J_{IS} = K^*[A]/[IS] \quad (1)$$

where [A] and [IS] are the blood concentrations of the two substances, and K is a constant.

The aim of this article is to test this hypothesis *in vitro*. The sodium cation, being a major charge carrier in the anode-to-cathode direction (i.e., from the body to the skin surface in the case of glucose monitoring), was selected as a candidate for the internal standard. Its concentration is constrained to the range 125 to 145 mM *in vivo*, and usually varies much less.¹² It satisfies a major criterion, therefore, for a suitable internal standard.

In a first set of experiments to explore validity of the IS idea, mannitol replaced glucose. Mannitol is nonmetabolizable, and is an ideal marker for the electroosmotic extraction of the endogenous sugar in that its molecular weight (182 Da) differs only slightly from that of glucose (180 Da), and it is otherwise very similar physicochemically. Subsequently, a second series of studies showed that glucose behaved very similarly to mannitol, and revealed that an ideal IS for the uncharged sugar would be a similar polar nonelectrolyte of comparable characteristics.

MATERIALS AND METHODS

Materials

[¹⁴C]-Mannitol (specific activity 51.0 mCi/mmol) was obtained from NEN Life Science Products (Paris, France), and D-[6-³H]-Glucose (specific activity 30.0 mCi/mmol) was purchased from Amersham Pharmacia Biotech (Orsay, France). D-Mannitol, D-Glucose, Tris base (α,α,α -Tris-(hydroxymethyl)-methylamine), sodium chloride, potassium chloride, and hydrochloric acid were analytical grade and purchased from Sigma Aldrich Co. (Saint Quentin Fallavier, France). Deionized water (resistivity >18.2 Mohm/cm²) was used to prepare all solutions.

Skin Preparation

Porcine ears were obtained less than 2 h after slaughter of the animal (Société d'Exploitation d'Abbatage, Annecy, France) and cleaned under running cold water. The whole skin was removed

carefully from the outer region of the ear and separated from the underlying cartilage with a scalpel. The tissue was then dermatomed to a 750- μm thickness (ZimmerTM Air Dermatome, Dover, OH) and cut into small squares ($\sim 9\text{ cm}^2$), which were wrapped individually in ParafilmTM and maintained at -20°C for no longer than 2 weeks before use.

Reverse Iontophoresis

For practical reasons related to the need for facile exchange of the subdermal solution (see below), side-by-side diffusion cells (transport area = 0.78 cm^2) were used in the iontophoresis experiments. The skin was clamped between the two half cells, with the stratum corneum side facing the cathodal chamber. Both chambers were initially filled with 25 mM Tris buffer pH 7.4 for a 1-h equilibration period. Chamber volumes were 1 mL for the anodal and 3 mL for the cathodal side.

Constant current (0.5 mA/cm^2) was applied for 6 hrs via Ag/AgCl electrodes connected to a custom-made power supply (Professional Design and Development Services, Berkeley, CA) controlled by LabviewTM software (National Instruments Inc., Austin, TX). All experiments were performed in quadruplicate, using skin samples from at least two different pigs.

In a first series of measurements, mannitol was used as a model for glucose. Its concentration in the subdermal compartment was varied over a range (3–10 mM) covering hypo- and hyperglycemic situations. Three experiments were performed, the details of which are summarized in Table 1.

Each began, after the 1-h equilibration period, with mannitol present in the subdermal (anodal) compartment at a nominally "normal" concentration of 5 mM, and "spiked" with $\sim 0.3\text{ }\mu\text{Ci/mL}$ of the ^{14}C -labeled sugar. The background electrolyte was 133 mM NaCl and 4 mM KCl in Tris buffer at pH 7.4; the solution initially present at the skin surface (cathodal chamber) was fresh 25 mM Tris buffer. The iontophoretic current was passed for 3 h, at which point the subdermal solution was changed according to the three protocols identified in Table 1. In experiments A and B, the background electrolyte remained fixed while the mannitol level was either doubled (experiment A), or first reduced to 3 mM, before being increased to 10 mM (experiment B). For experiment C, the mannitol concentration was increased, decreased, and then returned to 5 mM over a 3-h period. At the same time, the subdermal NaCl concentration was varied between its physiological limits as well passing from 133 to 125 mM, to 145 and then back to 133 mM. Each time that the subdermal solution was changed (i.e., every 60 min), the entire contents of the cathodal chamber were withdrawn (for analysis of mannitol and Na^+) and replaced with fresh buffer.

In a second set of measurements, the simultaneous reverse iontophoretic extraction of glucose and mannitol was studied. The subdermal solution always contained 10 mM sugar comprising glucose at concentrations of 10, 7, 5, 3, and 0 mM and mannitol levels of 0, 3, 5, 7, and 10 mM, respectively. ^3H -glucose was "spiked" into the solutions at $\sim 1\text{ }\mu\text{Ci/mL}$, ^{14}C -mannitol was added as before. The background electrolyte was pH 7.4 Tris buffer containing 133 mM NaCl and 4 mM KCl. After a

Table 1. Reverse Iontophoretically Extracted Fluxes (Mean \pm SD; $n = 4$) of Mannitol and Sodium during Three Experiments in Which the Subdermal Concentration Ratio of the Two Substances Was Varied

Experiment	Time (min)	[Mannitol] (mM)	[NaCl] (mM)	J_{Mannitol} ($\text{nmol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	$J_{\text{Na}^+}^a$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)
A	0–180	5	133	25.2 ± 4.3	10.1 ± 0.4
	180–360	10	133	63.2 ± 6.2	10.6 ± 0.6
B	0–180	5	133	27.8 ± 4.4	10.4 ± 0.7
	180–240	3	133	14.1 ± 2.8	9.7 ± 0.5
	240–300	10	133	48.2 ± 8.7	9.3 ± 0.4
C	0–180	5	133	28.8 ± 1.6	10.9 ± 0.3
	180–240	10	125	52.2 ± 4.1	10.7 ± 0.8
	240–300	3	145	15.4 ± 0.8	10.6 ± 0.3
	300–360	5	133	29.3 ± 0.8	11.4 ± 0.3

An analysis of variance shows no significant difference was found between sodium fluxes in experiment C.

^aIf all the measured J_{Na^+} are considered, an overall coefficient of variation of 6.9% is found.

1-h equilibration, current was passed and the cathodal chamber was sampled every hour as before for glucose, mannitol, and Na^+ . Each experiment, during which the subdermal solution remained unchanged, lasted 6 h.

Sample Analysis

From each 3-mL sample, a 1-mL aliquot was analyzed for sodium with an ion-selective electrode (RossTM Sure-Flow Sodium Combination Electrode, Orion Research Inc., Boston, MA) after addition of 1 mL of an ionic force adjustment solution (Orion Research Inc., Boston, MA). The remaining 2 mL were mixed with 5 mL of scintillation cocktail (Ultima Gold XR, Packard Instruments SA, Rungis, France) and then analyzed by liquid scintillation counting (LS 6500, Beckmann Instruments France SA, Gagny, France) for ^3H -glucose and/or ^{14}C -mannitol.

Statistical Analysis

The fluxes were determined as the mean \pm standard deviation (SD). Linear regressions were tested for significance by ANOVA. The constant K values for analyte extraction (eq. 1) were expressed as mean \pm confidence interval ($\alpha = 0.05$) and were compared with a Student's two-sided t -test.

RESULTS AND DISCUSSION

A first, important question to be addressed in this study was the constancy of the reverse iontophoretic extraction of Na^+ , the potential internal standard. The issue is particularly pertinent in diabetic patients because the systemic sodium concentration changes inversely with glucose levels. In hyperglycemia, the blood sodium level may fall to 125 mM, while in hypoglycemia, the concentration of this ion may reach 145 mM to maintain osmolarity.¹² The results in Table 1 and Figure 1 show that the iontophoretic extraction flux of Na^+ was independent of its subdermal level over the entire physiological range, and remained effectively constant while mannitol extraction responded proportionally to changes in its "systemic" concentration.

Sodium ions are transported by electromigration. The total charge transported in the iontophoretic system depends on the strength of the applied electric field and for how long it is applied. Given

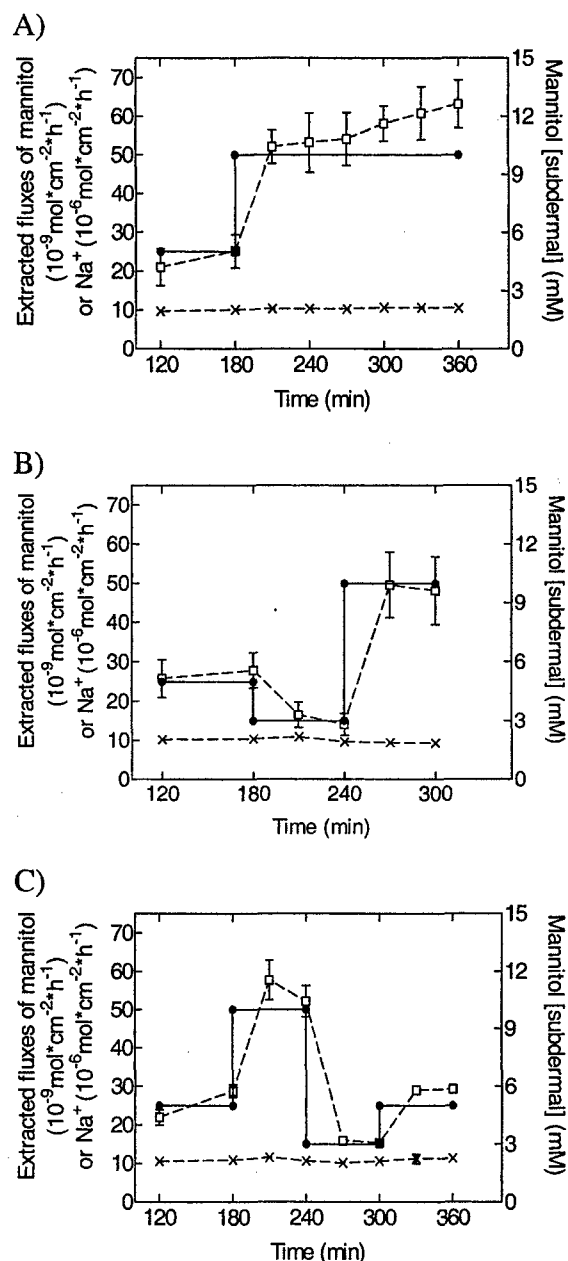


Figure 1. Simultaneous reverse iontophoretic extraction fluxes (mean \pm SD; $n = 4$) of mannitol (\square) and sodium (\times) as a function of time and subdermal mannitol concentration (\bullet) (see Table 1). Note that the standard deviations of the sodium fluxes are smaller than the symbol size.

that iontophoresis sets in motion a number of ions across the skin, the total current is shared among these different carriers. The fraction of the total current carried by a specific ion is called its transport number; the sum of the transport numbers of the ions moving in the circuit equals 1. According

to Faraday's law, the contribution of each ion to the total electrotransport is given by:

$$J_i = (t^*I)/(F^*z_i) \quad (2)$$

where J_i is the number of mol of the i th ion transported during a time t , z is the valence, F is Faraday's constant, and I is the total current flowing. Transport numbers are a complex function of the distribution and relative mobility of all the mobile ionic species in the membrane and are difficult to predict theoretically.⁴ The average sodium flux in this study was $10.3 (\pm 0.7) \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$, which corresponds to a transport number of 0.55 (i.e., sodium carried 0.79 out of a total charge transported of 1.44 Coulombs). The value of the Na^+ transport number agrees well with an earlier determination,⁵ for which excised human skin was used with a similar buffer and background electrolyte (though without K^+), and confirms that this ion is the major carrier of current across the membrane.

Here, the Na^+ reverse iontophoretic extraction flux did not change significantly despite either a 6% decrease or a 9% increase in the subdermal concentration. That is, we are not able to detect any redistribution of the responsibility for carrying the current when this moderate level of fluctuation in the "systemic" sodium concentration occurs. Perhaps this is not too surprising, given that, in the physiological milieu, Na^+ is present at a 30- to 100-fold higher concentration than those of its logical competitors, such as K^+ , Ca^{2+} , or Mg^{2+} . The result is also consistent with a previously published relationship between t_{Na^+} and concentration,¹³ which showed that the fraction of current carried by Na^+ increased with concentrations up to 50 mM, but then remained constant. An important next step, therefore, is to confirm this independence of t_{Na^+} *in vivo*.

Mannitol and glucose are neutral molecules and were transported by electroosmosis, the con-

vective movement of solvent that occurs through a charged membrane in response to the preferential passage of counterions when an electric field is applied. The isoelectric point of the pig skin model in this work is ~ 4.4 ¹⁴ (similar to human skin, ~ 4.8), therefore, at physiological pH, the membrane carries a net negative charge, and electroosmotic flow moves in the anode-to-cathode direction.⁵ Convective transport of a neutral molecule is directly proportional to its concentration, and the mannitol fluxes determined here conform to this behavior (Table 1 and Figure 1). It should be noted, however, that the mannitol extraction also "drifted" (usually) upwards when the subdermal concentration remained fixed for a relatively long time (see, e.g., Figure 1A). This may be due to an increase in the underlying passive permeability of the barrier as has been proposed in earlier work examining electroosmotic transport.¹⁵

The second series of experiments first confirmed that the results obtained for mannitol were excellent predictors of the electrotransport of glucose (Table 2). Despite the fact that the mannitol values are consistently, albeit slightly, higher than those for glucose (a fact attributed principally to the higher counting efficiency of ^{14}C compared to ^3H), these differences only attained statistical significance at a concentration of 7 mM (Table 2). It is noted that the radioactivity detected at the skin surface in the glucose experiments must correspond to glucose itself. Although metabolism of glucose is possible, of course, the metabolites, primary lactate, are negatively charged, and would therefore not be extracted towards the cathodal compartment.

It is worth noting that, with a subdermal concentration of 3 mM, the reverse iontophoretic extraction fluxes of mannitol and glucose after 6 h of current passage were $20.0 (\pm 2.3)$ and $19.7 (\pm 1.2) \text{ nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$, respectively. Recently, under similar experimental conditions, but using

Table 2. Reverse Iontophoretic Extraction Fluxes (Mean \pm SD; $n = 4$) of Glucose and Sodium (and Their Ratios) as a Function of Their Subdermal Concentration

Donor Concentration (mM)	Mannitol Flux ($\text{nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$)	Glucose Flux ($\text{nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$)	Ratio $J_{\text{glucose}}/J_{\text{Mannitol}}$
3	20.0 ± 2.3	19.7 ± 1.2	0.98
5	30.9 ± 3.3	28.6 ± 2.7	0.92
7	47.7 ± 2.9	41.3 ± 3.5	0.87
10	63.2 ± 6.2	60.5 ± 10.8	0.95

Only the values at 7 mM are significantly different ($p < 0.05$) between the two sugars. See text for further details.

a HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethansulfonic acid) buffer instead of Tris, and with a concentration of only 1 mM, the electroosmotic flux of mannitol was found to be $3.0 (\pm 1.0) \text{ nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$. Normalization with respect to concentration reveals that the convective solvent flux reported here is about twofold greater, presumably due to the differences in background electrolyte.

The second key objective was to test the internal standard hypothesis represented by eq. 1. The ratio of the extracted flux of mannitol or glucose to that of Na^+ (at each time point measured) was plotted against either the subdermal concentration of the corresponding sugar or against the ratio of the subdermal concentrations, $[\text{sugar}]/[\text{Na}^+]$. The excellent correlations (Figure 2) show that the hypothesis is validated and that the constant K in eq. 1 is truly constant. Similarity between the results for glucose and mannitol is reinforced by the fact that the respective K values are not statistically different (Table 3). With a knowledge of this constant, therefore, it follows that a measurement of the extracted flux ratio $J_{\text{glucose}}/J_{\text{Na}^+}$ permits the subdermal glucose concentration to be estimated, without the need for a blood sample, from the rearrangement of eq. 1:

$$[\text{Glucose}] = (J_{\text{Glucose}}/J_{\text{Na}^+}) \cdot ([\text{Na}^+]/K) \quad (3)$$

with $[\text{Na}^+]$ assumed to be fixed at a value of 133 mM.

The gradients of the plots in Figure 2B (i.e., the values of K) are much less than 1, reflecting the fact that the iontophoretic extraction of Na^+ is considerably more efficient than that of a neutral sugar. This is because the mechanisms of electrotransport of Na^+ and glucose are different: the former is electromigration, the latter electroosmosis. As the major charge carrier in the system, it is logical that Na^+ transport is driven most efficiently by the iontophoretic current.

Although it may be logical to expect that the magnitude of the solvent flow will be related to the electromigration of the principal cationic counterion across the skin, experimental evidence to

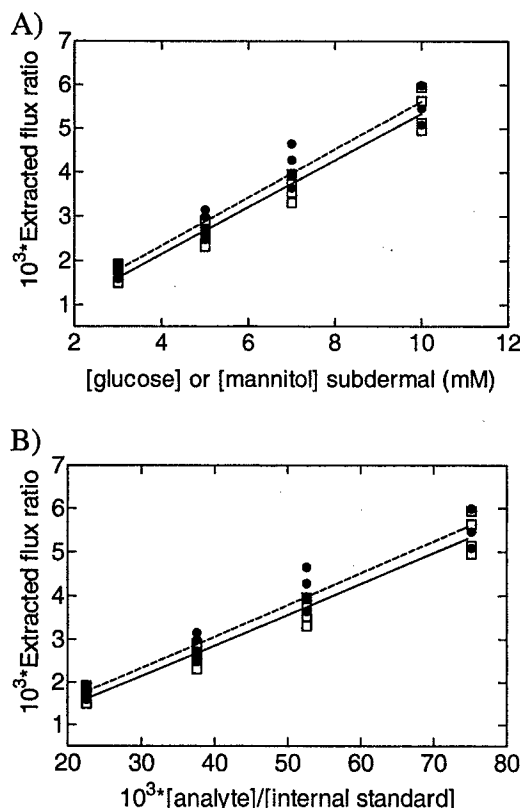


Figure 2. Ratio of the reverse iontophoretically extracted flux of mannitol (dashed line, ●) or glucose (solid line, □) to that of Na^+ as a function of (A) the subdermal concentration of the corresponding sugar, or (B) the ratio of the subdermal concentrations, $[\text{sugar}]/[\text{Na}^+]$. The linear regressions through the data points were: (A) for mannitol, $10^4 \cdot J_{\text{mannitol}}/J_{\text{Na}^+} = 1.55 + 5.46 \cdot [\text{mannitol}]$ ($r = 0.98$); for glucose, $10^4 \cdot J_{\text{glucose}}/J_{\text{Na}^+} = 0.23 + 5.31 \cdot [\text{glucose}]$ ($r = 0.98$); (B) for mannitol, $J_{\text{mannitol}}/J_{\text{Na}^+} = 0.16 + 0.073 \cdot ([\text{mannitol}]/[\text{Na}^+])$ ($r = 0.98$); for glucose, $J_{\text{glucose}}/J_{\text{Na}^+} = 0.02 + 0.071 \cdot ([\text{glucose}]/[\text{Na}^+])$ ($r = 0.98$).

support this contention is not readily to hand. Self-evidently, for a neutral analyte such as glucose, the ideal internal standard would be another uncharged compound, of similar physiological properties, whose systemic concentration,

Table 3. Values of the Proportionality Constant (K) (Mean \pm 95% Confidence Interval) between the Reverse Iontophoresis Extraction Flux Ratios of Analyte and Internal Standard and Their Corresponding Subdermal Concentration Ratio (eq. 1)

	Mannitol/ Na^+	Glucose/ Na^+	Glucose/Mannitol
K	0.073 ± 0.0019	0.072 ± 0.0027	0.87 ± 0.016
CV (%)	14.7	15.3	4.2
n	113	64	48

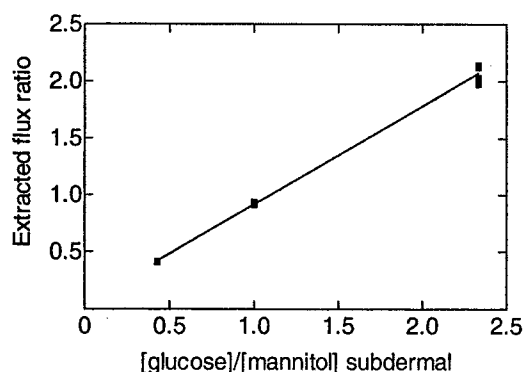


Figure 3. Ratio of the reverse iontophoretically extracted flux of glucose to that of mannitol as a function the ratio of their subdermal concentrations, [glucose]/[mannitol]. The linear regression through the data points was: $J_{\text{glucose/mannitol}} = 0.048 + 0.87 \cdot ([\text{glucose}]/[\text{mannitol}])$ ($r = 0.99$).

of course, is essentially invariant. We can use the experiments in which glucose and mannitol were both extracted and analyzed to demonstrate this point, taking mannitol as the internal standard. Equation 1 then becomes:

$$J_{\text{glucose}}/J_{\text{mannitol}} = K^*[\text{glucose}]/[\text{mannitol}] \quad (4)$$

Figure 3 plots these results graphically, and now we observe that K approaches the "ideal" value of unity (Table 3); the fact that $K = 0.87$ is probably due, as mentioned before, to the more efficient quantification of ^{14}C relative to ^3H , rather than an inherent difference between the electroosmotic extraction of glucose and mannitol. *In vivo*, of course, mannitol is not a practical internal standard, and a future goal is the identification of a sensible alternative.

In summary, the results presented here demonstrate the development of the reverse iontophoresis approach towards a truly noninvasive system that would not require calibration with a blood sample. This obvious advantage must be weighed against the need to identify a suitable internal standard and the additional analytical chemistry necessary for the practical quantification of two extracted substances. The concept demonstrated now needs to be confirmed *in vivo*, in human subjects. Specifically, it is now essential to determine whether (and how) K varies within and between subjects, as a function of skin site and condition. These objectives form the basis of research in progress. This work will also determine whether the apparently higher isoelectric point of human skin (relative to that of the pig)

leads to a lower convective solvent flow at pH 7.4. However, a lower ionic strength in the cathodal chamber, and a higher pH (~ 8 , e.g.), may compensate for this difference.¹⁶ Finally, although the present study has focused upon noninvasive glucose monitoring, the generality of the approach clearly suggests the use of the idea in other applications, such as therapeutic drug monitoring and other areas of clinical chemistry.¹⁷

ACKNOWLEDGMENTS

This work was supported by the "Programme commun de recherche en génie biomédical" of the EPFL and the Universities of Lausanne and Geneva, Switzerland, and by USAMRAA grant DAMD17-02-1-0712 (Fort Detrick, MD). The information presented does not necessarily reflect the position or the policy of the U.S. Government, and no official endorsement should be inferred.

REFERENCES

1. The Diabetes Control and Complications Trial Research Group. 1993. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes-mellitus. *N Engl J Med* 329: 977-986.
2. Koschinsky T, Heinemann L. 2001. Sensors for glucose monitoring: Technical and clinical aspects. *Diabetes Metab Res Rev* 17:113-123.
3. Sage BH. 1995. Iontophoresis. In: Smith EW, Maibach HI, editors. *Percutaneous penetration enhancers*. Boca Raton, FL: CRC Press, p 351-368.
4. Phipps JB, Gyory JR. 1992. Transdermal ion migration. *Adv Drug Del Rev* 9:137-176.
5. Burnette RR, Ongpipattanakul B. 1987. Characterization of the permselective properties of excised human skin during iontophoresis. *J Pharm Sci* 76: 765-773.
6. Guy RH. 1998. Iontophoresis—Recent developments. *J Pharm Pharmacol* 50:371-374.
7. Rao G, Glikfeld P, Guy RH. 1993. Reverse iontophoresis: Developpement of a non-invasive approach for glucose monitoring. *Pharm Res* 10:1711-1715.
8. Rao G, Guy RH, Glikfeld P, LaCourse WR, Leung L, Tamada J, Potts RO, Azimi N. 1995. Reverse iontophoresis: Non-invasive glucose monitoring in vivo in humans. *Pharm Res* 12:1869-1873.
9. Tamada JA, Garg S, Jovanovic L, Pitzer KR, Fermi S, Potts RO. 1999. Noninvasive glucose monitoring—Comprehensive clinical results. *JAMA* 282: 1839-1844.

10. Tamada JA, Bohannon NJV, Potts RO. 1995. Measurement of glucose in diabetics subjects using non-invasive transdermal extraction. *Nat Med* 1: 1198-1201.
11. Tierney MJ, Tamada JA, Potts RO, Eastman RC, Pitzer KR, Ackerman NR, Fermi S. 2000. The GlucoWatch biographer: A frequent automatic and noninvasive glucose monitor. *Ann Med* 32:632-641.
12. Hillier TA, Abbott RD, Barrett EJ. 1999. Hyponatremia: Evaluating the correction factor for hyperglycemia. *Am J Med* 106:399-403.
13. Yoshida NH, Roberts MS. 1994. Role of conductivity in iontophoresis. 2. Anodal iontophoretic transport of phenylethylamine and sodium across excised human skin. *J Pharm Sci* 83:344-350.
14. Marro D, Guy RH, Delgado-Charro MB. 2001. Characterization of the iontophoretic permselectivity properties of human and pig skin. *J Controlled Release* 70:213-217.
15. Kim A, Green PG, Rao G, Guy RH. 1993. Convective solvent flow across the skin during iontophoresis. *Pharm Res* 10:1315-1320.
16. Santi P, Guy RH. 1996. Reverse iontophoresis—Parameters determining electroosmotic flow: I. pH and ionic strength. *J Controlled Release* 38:159-165.
17. Delgado-Charro MB, Guy RH. 2003. Transdermal reverse iontophoresis of valproate: A non-invasive method for therapeutic drug monitoring. *Pharm Res* 20:1508-1513.

Noninvasive Glucose Monitoring by Reverse Iontophoresis in Vivo: Application of the Internal Standard Concept

AQ: A

ANKE SIEG,^{1,2} RICHARD H. GUY,^{1,2} and M. BEGOÑA DELGADO-CHARRO^{1,2*}

Background: The GlucoWatch® Biographer uses reverse iontophoresis to extract glucose across the skin to monitor glycemia in diabetes. The invasive daily calibration with a conventional "fingerstick" has been perceived as a disadvantage. We used an "internal standard" to render the approach completely noninvasive.

Methods: The simultaneous extraction of glucose and sodium by reverse iontophoresis was performed on human volunteers over 5 h, and blood glucose was measured in the conventional manner at each collection interval. These data were used for each volunteer to calculate an extraction constant (K), which equals the ratio of the extracted fluxes ($J_{\text{Glucose}}/J_{\text{Na}^+}$) normalized by the corresponding ratio of the concentrations in the blood ($[\text{Glucose}]/[\text{Na}^+]$). The values of K were compared between and within volunteers.

Results: The iontophoretically extracted glucose flux reflected the glucose concentration profiles in the blood, and sodium extraction remained essentially constant, consistent with the fact that its systemic concentration does not vary significantly. A constant value of K was established for two-thirds of the study population. However, the efficiency of glucose extraction varied seasonally, whereas the reverse iontophoresis of Na^+ did not; i.e., variation in K became apparent.

Conclusions: Use of the sodium ion as an internal standard might refine the determination of glycemia by reverse iontophoresis without requiring calibration with a blood sample.

© 2004 American Association for Clinical Chemistry

New techniques that measure analytes in the body non-invasively are subjects of considerable interest. The monitoring of blood sugar, in particular, is of great importance because of the large and growing population of people with diabetes who require regular and accurate information about their plasma glucose concentrations. The Diabetes Control and Complications Trial confirmed that blood glucose control can reduce the long-term complications of diabetes (1). Conventional self-testing methods require a blood sample, typically from the fingertip, a painful and inconvenient procedure with poor patient compliance. Consequently, there is considerable investment of resources at present in the development of noninvasive and continuous glucose monitoring technologies (2).

One such approach is iontophoresis, which uses a small electric current to drive charged and highly polar compounds across the skin at rates very much greater than their passive permeabilities. Two major transport mechanisms are involved: electromigration and electroosmosis. Electromigration is the movement of small ions across the skin under the direct influence of an electric field. Electron fluxes are transformed into ionic fluxes by the electrode reactions, and ionic transport proceeds through the skin to maintain electroneutrality. The total charge transported depends on the strength of the electric field and the duration of application. Iontophoresis sets in motion several ions across the skin, and all of them compete to carry a fraction of the current. The contribution of each ion to charge transport is called the transport number, the sum of which equals 1. According to Faraday's law, the flux of each ion in the iontophoretic circuit is given by:

$$J_i = \frac{t_i \times I}{F \times z_i} \quad (1)$$

where J_i is the flux of the i th ion, t_i is its transport number, and z_i is the valence; F is Faraday's constant; and I is the total current. Transport numbers depend on the relative

¹ University of Geneva, School of Pharmacy, Geneva, Switzerland.

² Centre Interuniversitaire de Recherche et d'Enseignement, "Pharmapépétides", Campus Universitaire, Archamps, France.

*Address correspondence to this author at: University of Geneva, School of Pharmacy, Quai Ernest-Ansermet 30, CH-1211 Geneva, Switzerland. Fax 33-450-95-28-32; e-mail Begonia.Delgado@pharm.unige.ch.

Received February 13, 2004; accepted April 30, 2004.

Previously published online at DOI: 10.1373/clinchem.2004.032862

mobilities and concentrations of all mobile ions in the iontophoretic system and, given that NaCl is the principal extracellular electrolyte in the body, Na^+ and Cl^- carry a major fraction of the current in iontophoresis.

Electroosmosis is the principal transport mechanism of uncharged molecules and of high-molecular-weight cations. The skin is negatively charged at physiologic pH and acts, therefore, as a permselective membrane to cations. This preferential passage of counterions induces an electroosmotic solvent flow that may carry neutral molecules in the anode-to-cathode direction. The volume flow, J_v [volume/(time · area)] is predicted (3) to be proportional to the potential gradient ($-d\Phi/dx$) established by the electric field:

$$J_v = L_{ve} \left(\frac{-d\Phi}{dx} \right) \quad (2)$$

where L_{ve} is the electroosmotic flow coefficient describing the direction and the magnitude of the volume flow. The molar flux of a solute j present at a molar concentration c_j is then:

$$J_j = J_v \times c_j \quad (3)$$

Electroosmosis depends on the charge on the membrane and may be modified by solutions (e.g., by their pH) that "couple" the electrodes to the skin, thereby changing the value of L_{ve} .

In clinical chemistry, iontophoresis has already been established as a tool used in the diagnosis of cystic fibrosis (whereby pilocarpine is administered to test the secretory function of the sweat glands) (4). The symmetry of iontophoresis renders it useful not only for the delivery of drugs, but also for the extraction of endogenous substances of clinical interest and, in particular, glucose (5).

This latter application has been investigated in depth and has led to the development of the GlucoWatch® Biographer (Cygnus Inc.) (6–10). The wrist-worn device monitors glucose continuously for up to 13 h, recording six glucose readings per hour. However, the device needs to be calibrated against blood glucose from the fingertip to correlate the extracted glucose amounts with subdermal concentrations. This essential step has been perceived as a disadvantage despite the fact that the GlucoWatch provides tremendously more information to the individual with diabetes than (the typical) one or two fingersticks per day. A completely noninvasive calibration approach would therefore be beneficial and would open the way to other applications of the reverse iontophoresis technology.

The concept addressed here is that of an internal standard (11). Because iontophoresis is nonspecific, many ions and small, uncharged species (in addition to the analyte of interest) are moved across the skin by the applied electric field. Instead of detecting uniquely the single target substance and calibrating its transdermal measurement by a blood assay, the idea is to monitor the extraction of two species simultaneously: the compound

of interest, the temporal change in concentration of which is of clinical importance (i.e., glucose), and a second analyte, the physiologic concentration of which is known and essentially fixed. If the iontophoretic transport of the analyte (A) and the latter, "internal standard" (IS), are independent of one another, then their fluxes (J) out of the skin should obey the relationship:

$$\frac{J_A}{J_{IS}} = K \times \frac{[A]}{[IS]} \quad (4)$$

where $[A]$ and $[IS]$ are the blood concentrations of the two substances, and K is a constant. The Na^+ ion, being a major charge carrier in the anode-to-cathode-direction, was selected as a potential internal standard. Previously, we have shown in vitro (11) that the relationship in Eq. 4 is obeyed even when the subdermal Na^+ concentration was allowed to vary over its maximum physiologic range (125–145 mmol/L).

The aim of the research described here is to test the internal standard hypothesis in vivo. The following questions were addressed: (a) what is the flux ratio $J_{\text{glc}}/J_{\text{Na}^+}$ in vivo, (b) can a common proportionality constant K be established for a subject population, (c) how accurate is the prediction of blood glucose when using the internal standard concept, and (d) what is the significance of inter- and intraindividual variability with respect to iontophoretic extraction? In addition, the potential utility of potassium as another cationic internal standard was examined.

Materials and Methods

STUDY POPULATION

Fourteen nondiabetic individuals (age range, 25–39 years; 4 males and 10 females) with no history of skin diseases participated in the study. Informed consent was obtained, and the study protocol had been approved by the internal review board of the University of Geneva in accordance with the principles outlined in the Declaration of Helsinki. In total, 42 experiments were performed: 8 individuals participated on a single occasion; the 6 other volunteers participated at least twice.

CHEMICALS

Tris base, NaCl, KCl, D-glucose, HCl, NaOH, and methanesulfonic acid were purchased from Sigma-Aldrich Co. and were at least of analytical grade. Deionized water (resistivity $>18.2 \text{ M}\Omega/\text{cm}^2$) was used to prepare all solutions.

IONTOPHORESIS

Two cylindrical glass cells (diameter, 1.6 cm; extraction surface, 2 cm^2) were fixed with foam tape (3M; Health Care) on each volunteer's ventral forearm with a distance of 7 cm between them. The anodal chamber was filled with 1.2 mL of 10 mmol/L Tris buffer (pH 8.5) containing 100 mmol/L NaCl; the cathodal chamber contained the

AQ: B

same volume of 10 mmol/L Tris buffer alone. Custom-made Ag/AgCl electrodes were inserted into the solutions and fixed 3–4 mm above the skin surface to ensure that no physical contact with the skin occurred. Direct current ($I = 0.6$ mA, current density = 0.3 mA/cm²) was passed for a total of 5 h and was controlled by a Phoresor II Auto (Iomed), a US Food and Drug Administration-approved constant current, iontophoretic power supply. Every 15 min after initiation of the current, the entire cathodal solution was collected and replaced by 1.2 mL of fresh buffer. The samples were immediately frozen until analysis.

After 2.5 h of iontophoresis, the volunteers ingested either a meal rich in carbohydrates or 75 g of glucose dissolved in 300 mL of water (Glucosum monohydricum, Pharmacopoeia Europea; H  nserler AG), so as to provoke a significant change in blood sugar. From this point onward, glycemia was measured before each subsequent 15-min collection interval by use of a conventional blood glucose monitor (Glucotrend 2; Roche Diagnostics). The "within-run" and "day-to-day" imprecision of the glucose monitor was $\leq 3\%$, and regression analysis of a comparison with an automated hexokinase reference method was:

AQ: C

y (mmol/L) = $0.98x + 0.08$ mmol/L (information from the supplier).

ANALYSES

Analytes were assayed separately by HPLC on an Ion Chromatograph 600 system (Dionex). Glucose was quantified by anion separation with pulsed amperometric detection on a gold electrode; Na⁺ and K⁺ were quantified by cation separation with suppressed conductivity detection. Calibration was performed with at least six external calibrators in each chromatographic run, covering linear ranges ($r^2 \geq 0.999$) of 0–30 μ mol/L for glucose, 0–5 mmol/L for Na⁺, and 0–1 mmol/L for K⁺. The limits of quantification (defined as a signal-to-noise ratio of 10) for glucose, Na⁺, and K⁺ were 0.15, 1.4, and 2.5 μ mol/L, respectively. The within-run imprecision (CV) of injection was 2.1% for a mean concentration of the analytes.

DATA ANALYSIS AND STATISTICS

Iontophoretic fluxes were calculated from the amounts extracted in each collection interval and plotted at the mid-time point of each interval; blood glucose concentrations were at the actual time of measurement.

Data are expressed as the mean (SD). Mean Na⁺ fluxes in each experiment were determined from 12 separate points. Electroosmotic volume flows were determined by normalizing the iontophoretic flux values of glucose by the corresponding blood concentrations; according to Eq. 3. Statistical differences were assessed by two-tailed Student *t*-test and ANOVA, followed by a Newman-Keuls multiple comparison test, calculated with GraphPad Prism 3.02 software. Individual values of *K* were determined by linear regression (extracted flux ratio vs blood concentration ratio), the significance of which was tested

by ANOVA; goodness-of-fit is given by r^2 . A common value of *K* was computed by pooled regression based on six experiments from different volunteers using analysis of covariance as described in Ref. (12). The accuracy of prediction was tested by plotting the predicted glucose values against the measured blood glucose in a Clark Error Grid (13).

Results and Discussion

LOCAL EFFECTS OF IONTOPHORESIS

All volunteers experienced a mild tingling sensation when the current was applied. The sensation was typically asymmetric, with more tingling felt at the anode than at the cathode. Generally, the sensation diminished with time of current application and lasted no longer than 30 min. Iontophoresis caused the skin sites beneath the electrode chambers to become slightly erythematous, an effect that disappeared within 24 h. In addition, a few, small punctuate lesions remained after the redness disappeared; these marks persisted for several days. These completely reversible effects are similar to those that have been reported in the literature (7) and do not appear to impair skin barrier function. In two individuals, after 60 min of current application, small blisters were seen beneath the electrode chambers, and the experiment was interrupted. The blisters subsequently diminished in size and disappeared completely within a few hours. It is believed that hypersensitivity to Tris buffer caused this reaction (14). These two individuals were excluded from further study.

GLUCOSE TRACKING

The iontophoretic transport of glucose, an uncharged, polar molecule, occurs by electroosmosis and is directly proportional to the subdermal concentration (3). However, a "warm-up" period is necessary to establish a pseudo-steady-state electroosmotic flow and to empty the glucose reservoir from the skin. The latter is attributable to local metabolism and is not reflective of glucose concentrations in the blood. The recommended warm-up period for the GlucoWatch G2 is 2 h, a period similarly adopted in this study. Subsequently, extraction every 15 min over the next 3 h allowed blood glucose tracking in 8 of the 12 volunteers studied. In these volunteers, electroosmotic flow was >5 μ L/h (Table 1). Fig. 1 shows the glucose extraction profiles for three volunteers who ingested on separate occasions either a carbohydrate-rich meal or 75g of a standard glucose load at 150 min. The glucose reverse iontophoretic extraction fluxes accurately followed the systemic glucose concentration. A time delay between the extraction rate and the blood concentration was apparent in those volunteers receiving the oral glucose load. Rapid absorption of glucose occurred with a higher peak value obtained (14 vs 10 mmol/L) relative to those volunteers who received a carbohydrate-rich meal. In contrast, ingestion of the carbohydrate-rich meal led to more gradual glucose absorption from the gastrointestinal

AQ: D

F1

Table 1. Mean (SD) normalized reverse iontophoretic glucose fluxes (with respect to the corresponding blood sugar concentration) and absolute values of sodium electrotransport in 12 volunteers.

Volunteer	Normalized glucose flux, $\mu\text{L/h}$	Na^+ flux, $\mu\text{L/h}$	K^a	r^2
1	10.9 (0.9)	11.8 (0.4)	0.137 (0.010) ^b	0.95
2	12.5 (0.7)	12.1 (0.2)	0.110 (0.012) ^b	0.90
3	10.5 (1.2)	12.8 (0.9)	0.114 (0.019) ^b	0.85
4	10.3 (1.4)	13.3 (0.3)	0.105 (0.107) ^b	0.80
5	9.4 (1.0)	11.8 (0.3)	0.136 (0.014) ^b	0.90
6	1.1 (0.2)	12.7 (0.5)	0.007 (0.003)	0.47 ^c
7	0.5 (0.1)	12.9 (0.3)	0.023 (0.007)	0.53 ^c
8	1.3 (0.5)	12.5 (0.6)	NS ^c	<0.1 ^c
9	1.0 (0.2)	10.7 (1.6)	NS ^c	<0.1 ^c
10	8.5 (1.2)	11.9 (1.0)	0.136 (0.014) ^b	0.90
11	8.4 (0.7)	13.2 (0.8)	0.280 (0.108)	0.57 ^d
12	11.2 (2.0)	12.7 (0.5)	NS	0.25 ^d

^a The constant K was determined from Eq. 4. The values in parentheses are the SE of the slope.

^b Results used to compute the mean, population value of K .

^c Efficiency of glucose extraction was poor, markedly increasing the error in the determination of glycemia from the iontophoretic data (see text). The values of K were not significantly different (NS) from zero.

^d For these individuals, blood sugar changes during the experiment were relatively small, such that the regression covered a rather narrow range (and yielded a poor r^2). Nevertheless, glucose tracking was accurate, and the data were therefore included in the Clark Error Grid analysis.

tract, such that differences between the plasma kinetics and the rate of extraction were blurred.

It is acknowledged that reverse iontophoresis samples the interstitial fluid (ISF) and that real differences exist between glucose kinetics in this compartment and those in the blood; this divergence is clearly relevant to the development of continuous glucose monitoring devices and the site(s) at which glucose is determined. For example, Aussedat et al. (15) have described a delayed response between increasing glucose concentrations in the interstitium compared with those in the blood in rats. On the other hand, a faster and more pronounced decrease in glucose concentrations in the interstitium was observed when blood sugar was lowered. This physiologic lag (estimated to be somewhat <4 min) was similarly observed in humans using the GlucoWatch (16). The possibility that insulin increases skin blood flow has also been reported (17), and this, in turn, might influence glucose extraction by reverse iontophoresis.

The warm-up period of 2 h of iontophoresis was sufficient to establish pseudo-steady-state electroosmosis. However, in volunteer 6 (see Fig. 1), an additional 45 min was necessary before glucose fluxes stabilized; from this point, good tracking of glycemia was observed.

Accurate glucose measurements by iontophoresis required efficient extraction. Only moderate or poor ($r^2 \leq 0.53$) correlation with plasma glucose was found when

the normalized glucose fluxes were <5 $\mu\text{L/h}$ (volunteers 6–9; Table 1); above this threshold, however, good to excellent ($r^2 \geq 0.80$) correlations were obtained (volunteers 1–5 and 10; Table 1).

CATION EXTRACTION

Reverse iontophoretic extraction fluxes of sodium are shown in Table 1 for all volunteers. These values stabilized 30 min after the initiation of iontophoresis (not shown) and remained essentially constant throughout the experiments even when significant changes in glycemia were occurring (see Fig. 1). The calculated mean (SD) transport number of sodium in these experiments was 0.55 (0.04). This is consistent with the in vitro results (11) and demonstrates that Na^+ is the principal charge carrier across the skin. Given that Na^+ is present in extracellular fluids at concentrations 30–50 times higher than those of other potential charge carriers, such as K^+ , Ca^{2+} , or Mg^{2+} , this result is not surprising. At this high concentration, Na^+ iontophoresis is effectively independent of its subdermal concentration over the physiologic range of 125–145 mmol/L and is unlikely to be affected by changes in the plasma concentrations of the other cations. Thus, alterations in the systemic Na^+ concentration during hypo- or hyperglycemia were not anticipated to affect the usefulness of Na^+ as an internal standard. The results from this study support this assumption in that reverse iontophoretic extraction of Na^+ was remarkably constant within and between individuals and was not significantly altered even when large excursions in glycemia occurred (see Fig. 1).

Potassium fluxes varied between individuals and within the same experiment (data not shown), being higher at the beginning of iontophoresis before gradually decreasing after 2 h of iontophoresis to values between 0.7 and 3.9 $\mu\text{mol/h}$; the calculated mean (SD) transport number was 0.07 (0.04), corresponding to a CV more than 10 times greater than that for sodium transport. Interestingly, the highest potassium fluxes were found in individuals with the lowest electroosmotic flux, an observation that needs to be confirmed in a larger population and for which (if real) the reason is unclear. The higher variability and lower transport number of potassium precluded its use for the calibration of glucose extraction, and no further work was pursued.

COMMON EXTRACTION CONSTANT (K)

The ratio of subdermal concentrations of glucose and Na^+ (assumed to be 133 mmol/L) were calculated and plotted against the corresponding ratio of extracted fluxes (Fig. 2). There was no systematic time delay between the blood concentration and extraction rate profiles. Linear regression (see Eq. 4) for each individual yielded an individual value of K , given in Table 1. These data are from six volunteers, for whom the normalized glucose flux was $\geq 8.5 \mu\text{L/h}$, and were used to determine a mean value of K .

F2

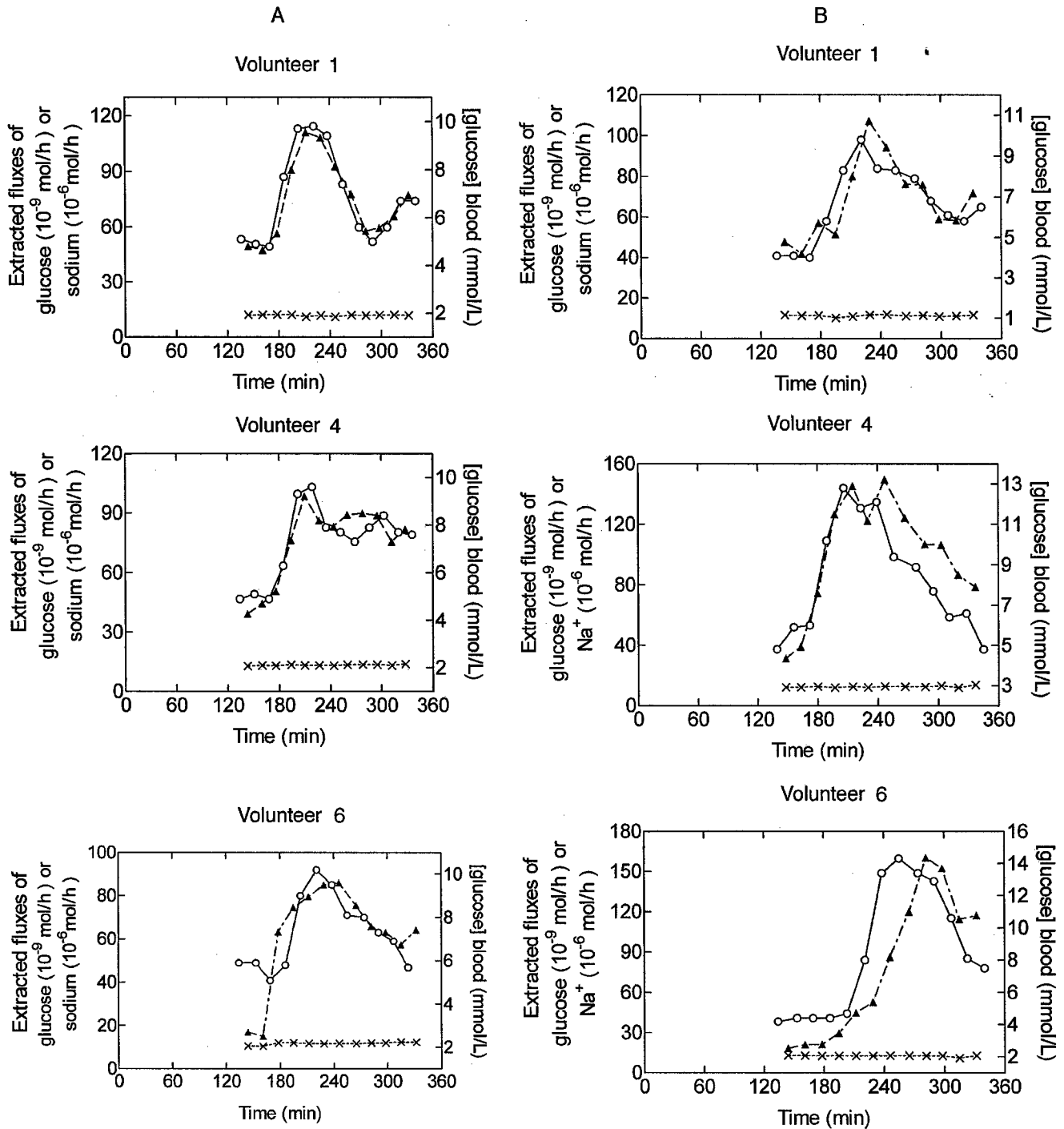


Fig. 1. Blood glucose profiles and reverse iontophoretically extracted glucose and sodium fluxes for three volunteers who ingested at 150 min a carbohydrate-rich meal (A) or a 75-g oral glucose load (B).

○, blood glucose concentration; ▲, glucose flux; ×, Na^+ flux.

AQ: F Analysis of covariance showed no significant differences in the individual K values. Pooled ANOVA yielded a common slope, i.e., the common mean extraction constant, namely, 0.12 (SD, 0.018).

PREDICTION OF BLOOD GLUCOSE

The mean K can be used to estimate blood glucose from the iontophoretic extraction flux data by rearrangement of Eq. 4:

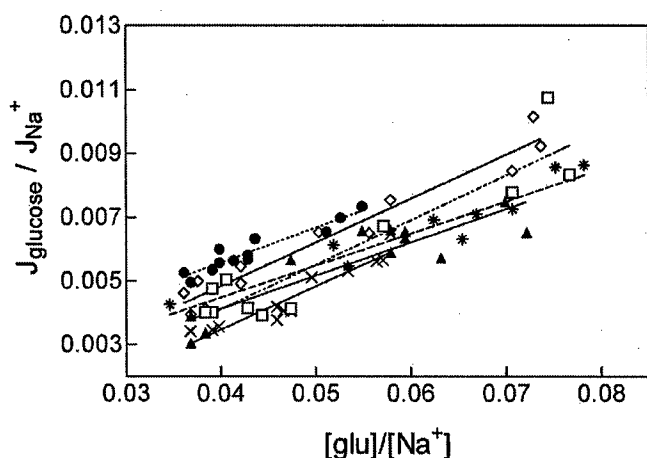


Fig. 2. Ratio of glucose to Na^+ extraction fluxes plotted against the corresponding ratio of glucose and Na^+ concentration in the blood (Eq. 4).

Lines of linear regression have been drawn through the data for six volunteers. The slopes and r^2 for each line are given in Table 1; the y-intercepts were in all cases less than ± 0.0020 .

$$[\text{Glucose}] = \frac{J_{\text{Glucose}}}{J_{\text{Na}^+}} \times \frac{[\text{Na}^+]}{K} \quad (1)$$

Data from an additional 16 experiments, in which electroosmosis was again $\geq 8.5 \mu\text{L/h}$, were analyzed in this way, and the estimated blood glucose concentrations were then compared with glycemia measured from the fingertip. The results, plotted as a Clark Error Grid, are shown in Fig. 3. Of a total of 181 data points, 142 (78.5%) fell in region A, and 39 (21.5%) fell in region B; that is, all results were in the clinical acceptable region A + B. It

F3

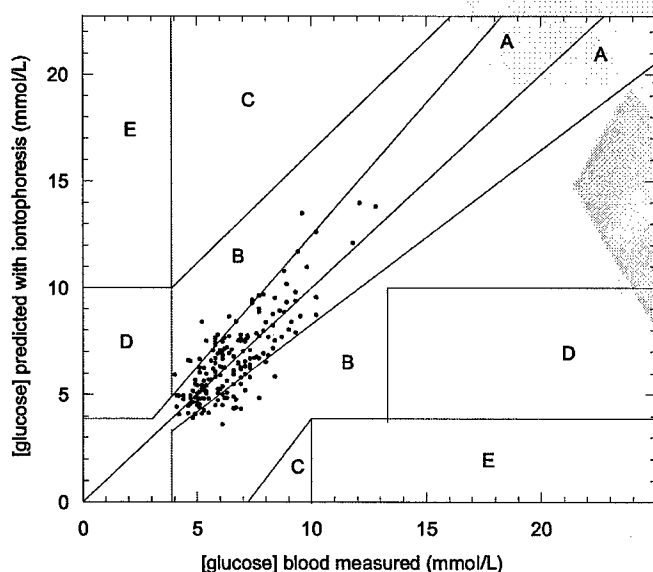


Fig. 3. Prediction of blood glucose values from iontophoretic flux data and the previously determined value of K from 16 additional experiments in volunteers 1–5 and 10–12.

The Clark Error Grid contains 181 data points with 78.5% in region A and 21.5% in region B.

must be accepted, however, that the range of glycemia covered by these experiments was limited: 4–13 mmol/L. Additional work is necessary to determine whether the predictions remain robust at more extreme degrees of hypo- and hyperglycemia. Linear correlation through all the data points in Fig. 3 yielded a slope (SE) of 0.96 (0.05) and an intercept (SE) of 0.10 (0.36). The correlation coefficient was 0.80. For this analysis, no attempt was made to temporally adjust the extraction profile to better overlap the corresponding blood glucose values, even when an apparent time lag could be clearly discerned (e.g., Fig. 1B). Although such a manipulation would have improved the goodness of fit for the determination of individual K values (and hence yielded a better blood sugar prediction for certain data points), a significant change in the absolute result is unlikely. This is because increasing glucose concentrations in the blood typically precede increases in the ISF concentrations, whereas decreasing blood concentrations lag behind those in the ISF (15). The objective here, in any case, was to analyze the results from the perspective of practical glucose monitoring, for which an individual's actual lag between blood and ISF concentration profiles is generally unknown.

AQ: G

AQ: H

INTER- AND INTRAINDIVIDUAL VARIABILITY

Glucose fluxes differed significantly in volunteers 6–9 from the rest of the population (see Table 1). Electroosmotic glucose transport was approximately an order of magnitude lower than that seen in the other volunteers. In contrast, essentially no differences in sodium extraction were found, with all values falling in the range of 10.7–13.3 $\mu\text{mol/h}$. This contrast is shown in Fig. 4. As a result, the K values for volunteers 6–9 were clearly smaller (Table 1), and the use of the previously discussed common K value would not yield accurate predictions of glycemia in these individuals.

F4

It might then be argued at this point that instead of

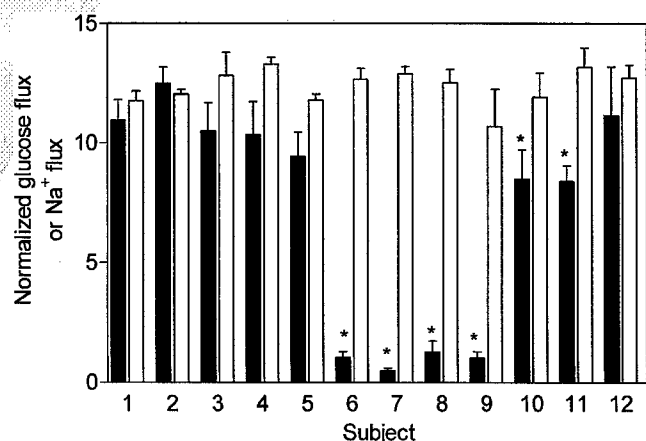


Fig. 4. Intersubject variability in normalized glucose extraction (in $\mu\text{L/h}$; ■) to reverse iontophoretic sodium transport flux (in $\mu\text{mol/h}$; □).

*, the normalized glucose fluxes from volunteers 5–11 were significantly lower than those of the other volunteers.

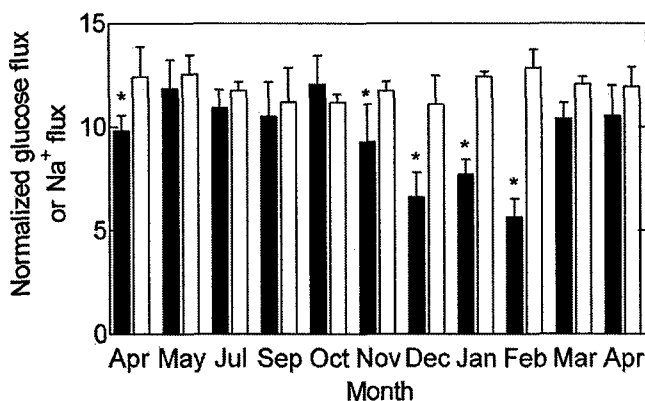


Fig. 5. Intrasubject (volunteer 1) variability in normalized glucose extraction flux as a function of time over a calendar year.

■, normalized glucose flux (in $\mu\text{L/h}$); □, sodium flux (in $\mu\text{mol/h}$). Significant decreases in glucose extraction (*, $P < 0.001$) were observed during the winter months (November through February).

attempting to use a common K for all individuals, K be determined for each individual in a separate calibration experiment. However, such an approach also demands that the within-subject variability as a function of time is reasonable. To test this hypothesis, we performed monthly experiments on volunteer 1 over the course of a calendar year. The results for the normalized glucose flux and the iontophoretic Na^+ extraction kinetics are shown in Fig. 5. Whereas Na^+ transport remained constant, significant decreases in electroosmosis were observed in the winter months. Additional data were also acquired in other volunteers (see Table 2) and revealed no clear pattern. Volunteers 2 and 10 behaved similarly to volunteer 1, whereas two other volunteers (3 and 4) showed no

seasonal effect; volunteer 6, in contrast, manifested a lower electroosmotic flow in the summer. In only two volunteers (4 and 10) were Na^+ fluxes significantly different, although the disparities would not have practical importance. Taken together, therefore, it appears that even an individual calibration for each individual might not permit an accurate prediction of glycemia at all times by the internal standard approach.

Although the fundamental reason for the variability in glucose extraction cannot be unequivocally deduced from this work, it can be concluded that a more appropriate internal standard will probably be another neutral substance that is transported by the same electroosmotic mechanism. In this way, any effects that change the charge on the skin (and/or its permselectivity) will similarly alter the extraction of glucose and the internal standard. The sodium ion, on the other hand, as the major charge carrier across the skin is not very sensitive to relatively subtle differences in skin charge. As such, it is a less than ideal internal standard for glucose. On the other hand, recent work has demonstrated the very appropriate use of Na^+ as an internal standard for the use of reverse iontophoresis as a noninvasive tool in the therapeutic monitoring of lithium. In this case, the normalization of lithium extraction flux with that of Na^+ gave improved prediction of the serum concentration of lithium in bipolar patients in vivo (18).

COMPARISON WITH IN VITRO DATA

The mean K measured in this work is somewhat higher than that measured in vitro with porcine skin (11); in that study, a mean electroosmotic flow of $5.1 \mu\text{L/h}$ together with a mean sodium flux of $8.0 \mu\text{mol/h}$ gave a constant of 0.07 (11). At least two factors may contribute to the better extraction in vivo: First, the electrode formulations were modified to maximize as far as possible the electroosmotic flow (e.g., pH 8.5 was used), and second, the presence of a functioning microcirculation provides for more facile access to the subdermal compartment. Particularly interesting, however, is that the marked variability in glucose extraction in vivo was not observed in vitro. At pH 7.4, electroosmosis varied no more than 30%, and a seasonal difference was not observed. A possible explanation may lie in the physiology of the skin appendages (e.g., the sweat glands), which have been recognized as important transport pathways in iontophoresis (19). Interindividual differences in appendageal morphology and effects of climate on function have been reported (20, 21) and may account for the greater variation observed in vivo as a function of ambient conditions. Additional work is clearly needed to better understand these observations.

In summary, this study demonstrates the development of the reverse iontophoresis approach to monitor glucose noninvasively without calibration with a blood sample. The internal standard hypothesis, using the Na^+ ion as the invariant endogenous calibrator, was confirmed for a

Table 2. Mean (SD) normalized reverse iontophoretic glucose fluxes (with respect to the corresponding blood sugar concentration) and absolute values of sodium electrotransport in six volunteers as an effect of season.

Volunteer	Season ^a	Normalized glucose flux, $\mu\text{L/h}$	Na^+ flux, $\mu\text{L/h}$	K^b	r^2
1	W	10.9 (0.9)	11.8 (0.4)	0.137 (0.010)	0.95
	S	6.6 (1.2) ^c	11.1 (1.4)	0.071 (0.007)	0.91
2	W	12.5 (0.7)	12.1 (0.2)	0.110 (0.012)	0.90
	S	9.0 (1.2) ^c	11.7 (0.2)	0.087 (0.021)	0.65
3	W	10.5 (1.2)	12.8 (0.9)	0.114 (0.019)	0.85
	S	9.7 (1.0)	12.5 (0.7)	0.083 (0.009)	0.91
4	W	10.3 (1.4)	13.3 (0.3)	0.105 (0.017)	0.80
	S	11.4 (2.4)	12.5 (0.5) ^d	0.140 (0.017)	0.87
6	W	1.1 (0.2)	12.7 (0.5)	0.007 (0.003)	0.47
	S	8.4 (3.3) ^c	12.6 (0.5)	0.125 (0.020)	0.79
10	W	8.5 (1.2)	11.9 (1.0)	0.136 (0.014)	0.90
	S	5.2 (1.0) ^c	12.6 (0.3) ^d	0.029 (0.004)	0.86

^a S, summer; W, winter.

^b The constant K was determined from Eq. 4. The values in parentheses are the SE of the slope.

^{c,d} Values measured during the winter were significantly different from those determined in summer: ^c $P < 0.0001$; ^d $P < 0.001$.

significant subset of the study population. However, Na^+ , being extracted by a different mechanism than glucose, did not reflect the entire range of variability in glucose extraction.

AQ: I This work was supported by the "Program commune de recherche en génie biomédical" of the EPFL, by the Universities of Lausanne and Geneva (Switzerland), by USAMRAA Grant DAMD17-02-1-0712 (Fort Detrick, MD), and by the US NIH (Grant EB 001420). The information presented does not necessarily reflect the position or the policy of the US government, and no official endorsement should be inferred.

References

1. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes-mellitus. *N Engl J Med* 1993;329:977-86.
2. Koschinsky T, Heinemann L. Sensors for glucose monitoring: technical and clinical aspects. *Diabetes Metab Res Rev* 2001;17:113-23.
3. Pikal JM. The role of electroosmotic flow in transdermal iontophoresis. *Adv Drug Del Rev* 1992;9:201-37.
4. Webster HL. Laboratory diagnosis of cystic fibrosis. *Crit Rev Clin Lab Sci* 1983;18:313-38.
5. Guy RH. Iontophoresis—recent developments. *J Pharm Pharmacol* 1998;50:371-4.
6. Rao G, Glikfeld P, Guy RH. Reverse iontophoresis: development of a non-invasive approach for glucose monitoring. *Pharm Res* 1993;10:1711-5.
7. Rao G, Guy RH, Glikfeld P, LaCourse WR, Leung L, Tamada J, et al. Reverse iontophoresis: non-invasive glucose monitoring in vivo in humans. *Pharm Res* 1995;12:1869-73.
8. Tamada JA, Bohannon NJV, Potts RO. Measurement of glucose in diabetics subjects using non-invasive transdermal extraction. *Nat Med* 1995;1:1198-201.
9. Tierney MJ, Tamada JA, Potts RO, Eastman RC, Pitzer KR, Ackerman NR, et al. The GlucoWatch biographer: a frequent automatic and noninvasive glucose monitor. *Ann Med* 2000;32:632-41.
10. Eastman RC, Chase HP, Buckingham B, Hathout EH, Feller-Byk L, Leptien A, et al. Use of the GlucoWatch Biographer in children and adolescents with diabetes. *Pediatr Diabetes* 2002;3:127-34.
11. Sieg A, Guy RH, Delgado-Charro MB. Reverse iontophoresis for noninvasive glucose monitoring: the internal standard concept. *J Pharm Sci* 2003;92:2295-302.
12. Zar JH. Biostatistical analysis, 2nd ed. Englewood Cliffs, NJ: Prentice-Hall, 1984:718pp.
13. Clark WL, Cox D, Gonder-Frederik LA, Carter W, Pohl SL. Evaluating clinical accuracy of systems for self-monitoring of blood glucose. *Diabetes Care* 1987;10:622-8.
14. Bohn S, Humn M, Bircher AJ. Contact allergy to trometamol [Short Communication]. *Contact Dermatitis* 2001;44:319.
15. Aussedat B, Dupire-Angel M, Gifford R, Klein JC, Wilson GS, Reach G. Interstitial glucose concentration and glycemia: implications for continuous subcutaneous glucose monitoring. *Am J Physiol Endocrinol Metab* 2000;278:E716-28.
16. Kulcu E, Tamada JA, Reach G, Potts RO, Lesho MJ. Physiological difference between interstitial glucose and blood glucose measured in human subjects. *Diabetes Care* 2003;26:2405-9.
17. Semé EH, Ijzerman RG, Gans ROB, Nijveldt R, de Vries G, Evertz R, et al. Direct evidence for insulin-induced capillary recruitment in skin of healthy subjects during physiological hyperinsulinemia. *Diabetes* 2002;51:1515-22.
18. Leboulanger B, Aubry JM, Bondolfi G, Guy RH, Delgado-Charro MB. Reverse iontophoretic monitoring of lithium in vivo [Abstract]. *Ther Drug Monit* 2003;25:499.
19. Cullander C, Guy RH. What are the pathways of iontophoretic current flow through mammalian skin? *Adv Drug Deliv Rev* 1992;9:119-35.
20. Sato F, Owen M, Matthes R, Sato K, Gisolfi CV. Functional and morphological changes in the eccrine sweat gland with heat acclimation. *J Appl Physiol* 1990;69:232-6.
21. Sato K, Sato F. Individual variations in structure and function of human eccrine sweat gland. *Am J Physiol* 1983;245:R203-8.

Electroosmosis in Transdermal Iontophoresis: Implications for Noninvasive and Calibration-Free Glucose Monitoring

Anke Sieg,^{*†} Richard H. Guy,^{*‡} and M. Begoña Delgado-Charro^{*‡}

^{*}University of Geneva, School of Pharmacy, 1211 Geneva, Switzerland; [†]Procter & Gamble, Egham TW20 9NW, United Kingdom; and [‡]Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, United Kingdom

ABSTRACT Reverse iontophoresis uses a small low electric current to noninvasively extract blood analytes, e.g., glucose, across the skin. The simultaneous quantification of the analyte extracted and of an additional endogenous substance of fixed and known concentration in the body permits the blood level of interest to be found without the need for an invasive calibration procedure. The transport phenomena underlying this approach, applied to glucose monitoring, has been investigated *in vitro*, using Na⁺ and neutral model solutes as endogenous "internal standards" (specifically, urea, glycerol, mannitol, and sucrose). The cathodal extracted fluxes of glucose under conditions of modified skin permselectivity were related to those of the different, potential internal standards. Flux ratios depended upon the iontophoretic conditions and the size of the neutral internal standards, whereas high variability was observed with Na⁺. Constant flux ratios were obtained with mannitol, glycerol, urea, and sucrose for which the mechanism of electrotransport was identical to that of glucose. The advantage of using a neutral internal standard, however, must be weighed against the need to identify and validate the marker under physiological conditions and the additional analytical chemistry necessary for the practical quantification of this substance.

INTRODUCTION

Human skin acts as a biological barrier, which renders transdermal drug delivery a significant challenge despite the skin's easy accessibility and large available surface. Iontophoresis enhances the delivery of charged and uncharged polar species via application of a low electric current (Kalia et al., 2004). The symmetry of iontophoresis makes the technique equally interesting, furthermore, for the clinical monitoring of drugs (Delgado-Charro and Guy, 2003; Leboulanger et al., 2004a,b,c) and biomarkers such as glucose (Glikfeld et al., 1989; Potts et al., 2002; Rao et al., 1995; Tamada et al., 1995; Tierney et al., 2001).

The total flux of a solute (J_i) during iontophoresis is the sum of electromigration (J_{EM}), convective flow (J_{EO}), and passive diffusion (J_p):

$$J_i = J_{EM} + J_{EO} + J_p. \quad (1)$$

Electromigration is the movement of small ions across the skin under the direct influence of the electric field. Electron fluxes are transformed into ionic fluxes via the electrode reactions; and ionic transport proceeds through the skin to maintain electroneutrality. The total charge transported depends on the strength of the electric field and the duration of application. Iontophoresis sets in motion a number of ions across the skin, and all of them compete to carry a fraction of the current. The contribution of each ion to charge transport is called its transport number, the overall sum of which equals 1. According to Faraday's law, the electromigration flux of each ion in the iontophoretic circuit is given by

$$J_{EM} = \frac{t_i \times I}{F \times z_i} \quad (2)$$

where t_i is the transport number, z_i is the valence of the i th ion, F is Faraday's constant, and I is the total current. The transport number depends on the ion's relative mobility (u_i) and charge z_i and upon its relative concentration c_i :

$$t_i = \frac{c_i \times z_i \times u_i}{\sum_{j=1}^n (c_j \times z_j \times u_j)} \quad (3)$$

Given that sodium and chloride ions are the principal extracellular electrolytes present in the body at high concentrations, they will invariably carry a major part of the current during iontophoresis *in vivo*.

Electroosmosis is the principal transport mechanism of uncharged molecules and of high molecular weight cations (Pikal, 1992). The skin is negatively charged at physiological pH, and acts therefore as a permselective membrane to cations. This preferential passage of counterions induces an electroosmotic solvent flow that carries neutral molecules in the anode-to-cathode direction. The volume flow J_V (volume \times time⁻¹ \times area⁻¹) is proportional to the potential gradient established by the electric field (Pikal, 1992)

$$J_V = L_{VE} \times \frac{-d\Phi}{dx} \quad (4)$$

where L_{VE} is the electroosmotic flow coefficient describing the direction and the magnitude of the volume flow resulting from the driving force, $-d\Phi/dx$. The electroosmotic flux

Submitted April 23, 2004, and accepted for publication August 23, 2004.

Address reprint requests to M. Begoña Delgado-Charro, Fax: 44-1225-386114; E-mail: b.delgado-charro@bath.ac.uk.

© 2004 by the Biophysical Society

0006-3495/04/11/3344/07 \$2.00

doi: 10.1529/biophysj.104.044792

contribution to the transport of a solute s present in the anodal compartment at molar concentration c_s is then

$$J_{EO} = J_v \times c_s \quad (5)$$

Electroosmosis assists the transport of high molecular weight cations and retards the passage of anions at pH 7. It can be modified by altering the permselectivity of the membrane and by manipulation of the formulation in the electrode chambers, that is by changing the value of L_{VE} (Santi and Guy, 1996a).

Passive diffusion of the solute j may be expressed as

$$J_p = D_j \times \frac{\Delta c_j}{h} \quad (6)$$

where D_j is the aqueous diffusivity of the solute, and $\Delta c_j/h$ represents its concentration gradient across the skin.

The contributions of electromigration, electroosmosis, and passive diffusion to the total iontophoretic flux depend on the structure and physicochemical properties of the species being transported. For small ions such as Na^+ or Cl^- , electromigration dominates; on the other hand, neutral solutes are transported by electroosmosis and (usually) to a much lesser extent by passive diffusion.

The reverse iontophoretic extraction of glucose across the skin is primarily electroosmotic and has been successfully used to monitor glycemia in diabetics (GlucoWatch Biographer, Cygnus, Redwood City, CA) (Potts et al., 2002; Tamada et al., 1995; Tierney et al., 2001). The commercially available wrist-worn device tracks glucose continuously for up to 13 h, making six measurements per hour. However, because the glucose extraction efficiency varies significantly within and between patients, the device must be calibrated against a conventional fingertip blood glucose reading before each use. This essential step has been perceived as a disadvantage despite the fact that the GlucoWatch provides tremendously more information to the diabetic than one or two "finger-sticks" per day.

The long-term objective of the research presented here is to refine the iontophoresis technology so as to avoid the invasive calibration step. The use of an internal standard has been proposed as a strategy to attain this goal. As iontophoresis is nonspecific, many ions and small uncharged species (in addition to the analyte of interest) are moved across the skin. Instead of detecting uniquely the single target substance extracted by iontophoresis and calibrating its transdermal measurement via a blood assay, we propose to monitor the extraction of two species simultaneously: the compound of interest, the temporal change in the concentration of which is of clinical importance (i.e., glucose), and a second analyte, the physiological concentration of which is known and essentially fixed. If the iontophoretic transport of the analyte (A)

and the latter internal standard (IS) are independent of one another, then their fluxes (J) out of the skin should obey the relationship

$$\frac{J_A}{J_{IS}} = K \times \frac{[A]}{[IS]} \quad (7)$$

where $[A]$ and $[IS]$ are the blood concentrations of the two substances, and K is a constant. Previous work (Sieg et al., 2003) established the validity of this equation in vitro, in a series of experiments in which glucose and sodium concentrations were varied over their clinically relevant ranges. However, subsequent in vivo studies (Sieg et al., 2004) found much more variability in glucose extraction, both within and between subjects (Sieg et al., 2004). As a consequence, the "constant" K was only truly constant for about two-thirds of the population.

A first objective of the work described here, therefore, is to understand the in vitro-in vivo differences that have been observed. Further in vitro experiments have been performed under conditions known to modify the skin's net charge (i.e., its permselectivity). Specifically, by manipulating the pH on either side of the barrier (Santi and Guy, 1996a; Marro et al., 2001), it was possible to investigate whether the iontophoretic extraction of Na^+ and glucose varied in parallel and to assess the impact of these changes on the constancy of the parameter K in Eq. 7. A second objective was to examine different, small, neutral solutes as alternative internal standards for glucose extraction. In this case, both analyte and internal standards were moved across the skin by electroosmosis. So as to evaluate the robustness of this approach, electroosmosis was modified to different degrees using different background electrolytes (Santi and Guy, 1996b).

MATERIALS AND METHODS

Chemicals

D-glucose, Tris base (α, α, α -Tris-(hydroxymethyl)-methylamine), sodium chloride, potassium chloride, D-mannitol, urea, glycerol, D-sucrose, calcium chloride, EDTA, sodium hydroxide, and hydrochloric acid were analytical grade and purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). D-[1- ^{14}C]-mannitol, [^{14}C]-urea, [^{14}C (U)]-glycerol, [^{14}C (U)]-sucrose (specific activities 51.5, 54.3, 142.7, and 401.0 mCi/mmol, respectively) were obtained from PerkinElmer Life Sciences, Rungis, France), and D-[6- ^3H]-glucose (specific activity 35.0 mCi/mmol) was purchased from Amersham Pharmacia Biotech (Orsay, France). Deionized water (resistivity $>18.2 \text{ MOhm/cm}^2$) was used to prepare all solutions.

Skin preparation

Porcine ears were obtained $<2 \text{ h}$ after slaughter of the animal (Société d'Exploitation d'Abbatage, Annecy, France) and cleaned under running cold water. The whole skin was removed carefully from the outer region of the ear and separated from the underlying cartilage with a scalpel. The tissue was then dermatomed to a thickness of $750 \mu\text{m}$ (Zimmer Air Dermatome, Dover, Ohio) and cut into small squares ($\sim 9 \text{ cm}^2$), which were wrapped individually in Parafilm and maintained at -20°C for no longer than 2 weeks before use.

Reverse iontophoresis

Side-by-side diffusion cells (transport area = 0.78 cm^2) with three compartments representing the anodal, subdermal, and cathodal chambers (Leboulanger et al., 2004b) were used in the iontophoresis experiments. Volumes were 1.5 ml for the electrode chamber and 3.5 ml for the subdermal compartment.

A piece of excised skin was clamped between the central compartment and each electrode chamber, with the dermal surface facing the central compartment, and the cell was assembled as shown in Fig. 1. The background buffer, 10 mM Tris, was used for all experiments at pH 6.3, 7.4, or 8.5. All chambers were initially filled with this buffer for a 1-h equilibration period. After replacing the buffer by the appropriate electrode and subdermal solutions, constant current (0.5 mA/cm^2) was applied for 6 h via Ag/AgCl electrodes connected to a constant current power supply (KEPCO APH-1000DM, KEPCO Inc., Flushing, NY). After each hour, the current was interrupted, and the entire contents of anodal and cathodal chamber were withdrawn and replaced by fresh receiver solution. All experiments were performed in quadruplicate, using skin samples from four different pigs. The fluxes shown correspond to the 5–6th h of iontophoresis; steady values were typically attained after 3 h of current passage.

Glucose and sodium extraction at different pH

In these experiments, the subdermal (donor) solution comprised 10 mM glucose, 133 mM NaCl, and 4 mM KCl in Tris buffer at pH 6.3, 7.4, or 8.5, respectively. The anodal compartment contained 100 mM NaCl in Tris buffer, the cathodal compartment Tris buffer alone at the same pH as the donor solution. A control experiment with pH 7.4 in the donor and pH 8.5 in the electrode chambers was also performed.

Simultaneous extraction of neutral compounds

To study the simultaneous extraction of glucose and model neutral compounds, the subdermal solution contained 133 mM NaCl, 4 mM KCl, 5 mM glucose, and either urea, glycerol, mannitol, or sucrose, (again, at a concentration of 5 mM); further, the solution was spiked with $\sim 0.2 \mu\text{Ci/ml}$ of the corresponding ^{14}C -isotope and $\sim 0.5 \mu\text{Ci/ml}$ of ^3H -glucose. The pH of the subdermal solution was 7.4 for all experiments except for the measurements at pH 6.3 when this lower pH was also maintained in the central compartment (see Results and Discussion). In all experiments, the anodal and cathodal solutions were identical and comprised 10 mM Tris buffer at pH 6.3

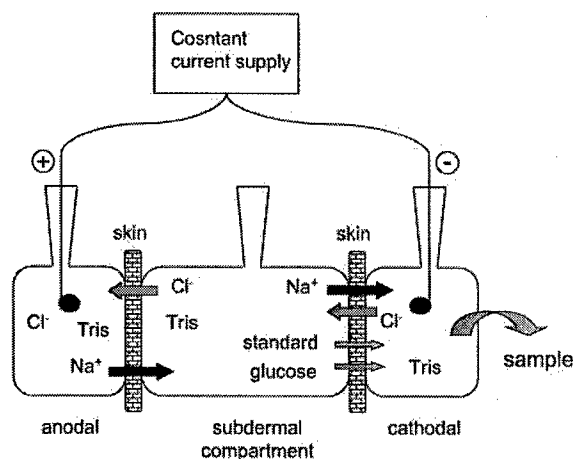


FIGURE 1 Schematic view of the setup for the reverse iontophoresis experiments.

or pH 8.5 together with additional electrolytes as described below. Passive controls, following the same experimental procedure without current application, were performed for the experiments with urea and glycerol.

Sample analysis

Samples from the first series of measurements were quantified by high-performance ion chromatography using a Dionex IC 600 system (Dionex, Sunnyvale, CA). Glucose was assayed by anion separation with pulsed amperometric detection; Na^+ was quantified by cation separation with suppressed conductivity detection (Sieg et al., 2004).

For the experiments using radioactivity, samples from the electrode chambers were mixed with 5 ml scintillation cocktail (Ultima Gold XR, PerkinElmer Life Sciences) and then analyzed by liquid scintillation counting (LS 6500, Beckmann Instruments France SA, Gagny, France) for ^3H -glucose and the ^{14}C -isotope from the second neutral compound.

Statistical analysis

Iontophoretic fluxes and values of K were determined as the mean \pm SD and were compared with a one-way ANOVA followed by Tukey's Multiple Comparison Test. Excel MS Windows 97 and GraphPad Prism 3.02 Software (GraphPad Software, San Diego, CA) were used for data analysis.

RESULTS AND DISCUSSION

As earlier explained, at conditions close to physiological pH, electroosmosis is predominantly in the anode-to-cathode direction. All solutes investigated were much more efficiently extracted, therefore, at the cathode, and attention is focused specifically on these data alone.

pH dependence of glucose and Na^+ transport

By modification of the pH, we aimed to gradually change the skin's permselectivity and hence to modify the extraction of glucose by electroosmosis. If Na^+ electromigration was affected in a similar manner, then we would expect the extraction constant K (Eq. 7) to remain essentially constant.

Although the pH and composition of the subdermal milieu *in vivo* cannot be manipulated, it is possible to perform rather specific modifications in the *in vitro* experimental setup. To avoid the additional complication of a pH gradient across the skin, therefore, it was decided initially to vary the pH on both sides of the skin in systematic steps of 1.1 pH units between 8.5 and 6.3. In this way, the skin retained its cation permselectivity (the isoelectric point of porcine ear skin having been shown to be ~ 4.4 (Marro et al., 2001)), and the variability in electroosmotic behavior observed with a significant pH gradient across the skin (Kim et al., 1993) was precluded.

The skin's permselectivity depends strongly on the pH of the surrounding media (Marro et al., 2001; Phipps and Gyory, 1992). With a change in the fixed charge on the membrane, the preferential passage of cations and the extent of electroosmotic flow can be modified. Glucose and Na^+ fluxes after 6 h of iontophoresis at different pHs are shown in Table 1.

TABLE 1 Simultaneous glucose and Na⁺ extraction fluxes and the deduced sodium transport number (t_{Na}^+), together with calculated proportionality constant K (Eq. 7), as a function of pH

pH subdermal/electrodes	Glucose flux (nmol \times cm ⁻² \times h ⁻¹)	Na ⁺ flux (μ mol \times cm ⁻² \times h ⁻¹)	t_{Na}^+	K
6.3/6.3	27.4 \pm 8.6	8.0 \pm 0.3	0.43 \pm 0.01	0.045 \pm 0.014
7.4/7.4	54.3 \pm 5.5	10.7 \pm 0.7	0.57 \pm 0.02	0.068 \pm 0.007
8.5/8.5	56.8 \pm 4.0	11.5 \pm 1.2	0.62 \pm 0.07	0.066 \pm 0.005
7.4/8.5	56.4 \pm 9.4	11.7 \pm 0.7	0.62 \pm 0.04	0.064 \pm 0.007

Although no differences were observed when the pH was changed from 7.4 to 8.5, both glucose and sodium electrotransport were reduced significantly when the pH was decreased to 6.3. However, the amplitude of this modification was not the same for the two solutes. Whereas sodium fluxes decreased to $\sim 75\%$ of its value at pH 7.4 (that is, from 10.7 (± 0.7) to 8.0 (± 0.3) $\mu\text{mol} \times \text{cm}^{-2} \times \text{h}^{-1}$), electroosmotic transport of the glucose decreased by one half (from 54.3 (± 5.5) to 27.4 (± 8.6) $\text{nmol} \times \text{cm}^{-2} \times \text{h}^{-1}$). Given that the subdermal glucose and Na⁺ concentrations were kept constant, the proportionality constant K (calculated from Eq. 7) fell significantly from 0.068 ± 0.007 to 0.045 ± 0.014 . Note that the values for Na⁺ extraction are 2–3 orders of magnitude higher than those for glucose; in other words, sodium electromigration is much more efficient than glucose extraction by electroosmosis at concentrations close to physiological conditions. This difference is reflected in the value of the constant K , which is < 0.1 .

This differential modification of electromigration and electroosmosis across the skin has been alluded to previously (Marro et al., 2001; Phipps and Gyory, 1992). The transport number of Na⁺ in iontophoresis under normal physiological conditions (~ 0.6 at pH 7.4 (Phipps and Gyory, 1992)) reflects the net negative charge on the skin and is significantly higher than in simple aqueous solution (~ 0.4) (Burnette and Ongpipattanakul, 1987). Similarly, because of this cationic permselectivity, electroosmosis proceeds in the anode-to-cathode direction, achieving $\sim 1 \mu\text{l} \times \text{cm}^{-2} \times \text{h}^{-1}$ at neutral pH (Burnette and Ongpipattanakul, 1987; Marro et al., 2001). However, if the pH of the solution bathing the skin is lowered to 4, while the Na⁺ transport number decreases by $\sim 40\%$, electroosmotic flow is completely attenuated from the anode and now occurs in the opposite, cathode-to-anode, direction (Marro et al., 2001). That is, alteration of the net charge on the skin has a far more dramatic impact on convective flow than on electromigration. For reverse iontophoresis, it is clear that the high subdermal concentration of NaCl guarantees that Na⁺ will dominate the carrying of current toward the cathode, regardless of the level of negative charge of the skin; on the other hand, changes in the latter parameter will result in significant changes in electroosmosis.

In previous experiments in vivo in man, Na⁺ transport numbers from 0.48 to 0.64 were determined at pH 8.5 ($I = 0.6$ mA) (Sieg et al., 2004). Normalized glucose fluxes ranged from 0.25 to 6.25 $\mu\text{l} \times \text{cm}^{-2} \times \text{h}^{-1}$, and the inferred constant K varied between 0.01 and 0.13. Compared to the results from

this investigation, the Na⁺ transport numbers were in good agreement: average values in vitro and in vivo 0.60 vs. 0.55, respectively). Notably, this overlap was found despite the facts that i), the in vitro subdermal solution did not contain all physiologically relevant ions, and ii), porcine rather than human skin was used. The normalized glucose flux at pH 8.5 in vitro was $5.7 \mu\text{l} \times \text{cm}^{-2} \times \text{h}^{-1}$ ($I = 0.4$ mA), and is comparable to the upper value observed in vivo. It is important to emphasize that the in vivo experiments were performed with a slightly higher current, and that the up to 10-fold difference in glucose fluxes was not observed in vitro. To correct for these important interindividual differences that are not mirrored by the Na⁺ extraction flux, attention was next focused on the identification of a potential neutral internal standard transported by the same mechanism as glucose, namely electroosmosis.

Simultaneous extraction of neutral solutes and glucose

In examining the transport behavior of the neutral internal standard candidates relative to that of glucose, it was particularly interesting to determine the flux ratios under different conditions of skin permselectivity. In addition to exogenous factors, such as the composition of the electrode formulations and the current applied, iontophoretic extraction in vivo depends on a number of biological factors (including net charge on the skin, local blood flow, etc.) that are difficult to control and modulate in vitro. Nevertheless, the electrical properties of the skin can be modified in vitro by changing the pH, as before, and by the use of different background electrolytes, and this was the strategy adopted here.

The candidates for the internal standard were chosen based upon their molecular weight (MW, ranging from 60 to 342), biological relevance (urea and glycerol are present at sufficiently high concentrations in the blood to be extracted by reverse iontophoresis in amounts sufficient for relatively straightforward analysis), and similar physicochemical properties (glycerol, mannitol, and sucrose having multiple-OH functions like glucose). The cathodally extracted fluxes of glucose and the model solutes at 6 h are shown in Table 2. For urea and glycerol, passive diffusion contributed significantly to the overall transport, and the uniquely electroosmotic contribution (J_{EO}) was therefore calculated after subtracting passive transport from total measured flux. If extraction into

TABLE 2 Cathodal extraction fluxes (nmol \times h⁻¹ \times cm⁻²) at 6 h for glucose and the neutral internal standard candidates

	MW	pH 8.5 + 30 mM NaCl	pH 8.5 + 30 mM NaCl + 10 mM EDTA	pH 8.5 + 100 mM CaCl ₂ *	pH 6.3 + 30 mM NaCl*	n
Urea	60	41.4 \pm 7.9 [†]	52.0 \pm 6.6 [†]	26.5 \pm 10.5 [†]	27.2 \pm 7.9 [†]	4
<i>J</i> _{EO}		33.2 \pm 3.6	46.5 \pm 3.9	20.1 \pm 6.2	25.5 \pm 6.8 [†]	4
Glycerol	92	36.7 \pm 8.5 [†]	49.6 \pm 9.2	21.7 \pm 11.8	16.1 \pm 6.5	4
<i>J</i> _{EO}		33.9 \pm 7.9	47.4 \pm 8.4	18.7 \pm 9.4	14.2 \pm 5.4	4
Mannitol	182	28.2 \pm 2.6	41.5 \pm 4.7	14.7 \pm 2.6	11.8 \pm 4.4	4
Glucose	180	26.2 \pm 3.7	39.6 \pm 5.0	15.2 \pm 5.5	12.4 \pm 4.1	16
Sucrose	342	27.2 \pm 5.0	40.8 \pm 6.0	16.0 \pm 5.8	12.6 \pm 6.7	4

The electroosmotic contributions (corrected for passive diffusion) for urea and glycerol fluxes are given as *J*_{EO}.

*The extraction fluxes at pH 8.5 + 100 mM CaCl₂ were not significantly different, for any solute, from those at pH 6.3 + 30 mM NaCl.

[†]These values are significantly different (*p* < 0.01) from all others in the corresponding columns.

a solution at pH 8.5 in the presence of a relatively low level of background electrolyte is considered as a reference value, addition of 10 mM EDTA significantly increased electroosmosis. On the other hand, supplementing the formulation with 100 mM CaCl₂ decreased convective transport by 50%. A comparable reduction was induced by simply lowering the pH of the cathode solution to 6.3, an effect already observed in the first part of this study. Calcium has been shown to shield the skin's net negative charge (presumably via an interaction with carboxylic acid groups in the skin (Phipps and Gyory, 1992)). Conversely, EDTA has been suggested to increase the skin's permselectivity by complexing endogenous divalent cations, such as Ca²⁺, thus enhancing electroosmosis toward the cathode (Santi and Guy, 1996b). It is noted that, for all solutes (except urea at pH 6.3), convective flow was essentially the same at each set of experimental conditions studied. Although there is evidence of molecular hindrance (i.e., a sieving effect as the convective volume flow passes through narrow channels) under certain iontophoretic conditions (Ruddy and Hadzija, 1992), the results described here are consistent with the hypothesis that electrotransport via electroosmosis is relatively constant for low molecular weight solutes (Pikal, 1992).

In the second part of the study, the subdermal concentration of all the neutral solutes considered was 5 mM. Under these conditions, according to Eq. 7, the flux ratio (glucose/internal standard) must equal the constant *K*, which represents, therefore, the relative efficiency of glucose transport to that of the candidate internal standard. Thus, if *K* is greater than unity, glucose transport is higher than that of the standard, whereas *K* < 1 indicates that the standard is more efficiently extracted than glucose. Modification of skin permselectivity

had a significant impact on *K* (Table 3, Fig. 2), the values of which increased gently with increasing molecular weight. Not surprisingly, mannitol, as an isomer of glucose, was reverse iontophoretically extracted at the same rate as glucose, yielding *K* values close to unity under all experimental conditions. This behavior is entirely consistent with an earlier in vitro study (Sieg et al., 2003). Urea (MW 60) transport was significantly higher than that of glucose, and *K* values, which differed significantly between the iontophoretic conditions, were consistently <1. Glycerol (MW 92) showed behavior intermediate between mannitol and urea. Finally, sucrose (having the highest MW, nearly double that of glucose) yielded values of *K* sensitive to the composition of the cathode formulation. At pH 8.5, in the presence or not of EDTA, *K* was close to 1; on the other hand, at high calcium concentration and at lower pH, *K* equaled 1.2.

Although the values of *K* determined in this series of experiments, over all solutes and all cathodal solution conditions, differed by no more than a factor of 2, (i.e., ranging from ~0.6 at the lowest for urea up to ~1.2 at the highest for sucrose), the trend in Fig. 2, with respect to molecular weight, deserves further discussion. The *K* values reported were determined from Eq. 7 using the absolute fluxes measured at 6 h. Although these fluxes have no electromigration contribution (Eq. 1), they do include passive diffusion as well as electroosmosis. As mentioned above, the passive transport of urea and glycerol did contribute significantly to the total and it might be anticipated, therefore, that this phenomenon would explain, at least in part, why *K* deviates from unity. That is, when glucose and the internal standard are present subdermally at equal concentrations

TABLE 3 Calibration constant *K* (glucose/internal standard) (*n* = 4)

Internal standard	MW	pH 8.5 + 30 mM NaCl	pH 8.5 + 30 mM NaCl + 10 mM EDTA	pH 8.5 + 100 mM CaCl ₂ *	pH 6.3 + 30 mM NaCl*
Na ⁺	23	0.064 \pm 0.007	-	-	0.045 \pm 0.014
Urea	60	0.61 \pm 0.04	0.71 \pm 0.05	0.51 \pm 0.11	0.47 \pm 0.03
Glycerol	92	0.75 \pm 0.05	0.79 \pm 0.05	0.71 \pm 0.06	0.74 \pm 0.03
Mannitol	182	0.92 \pm 0.01	0.92 \pm 0.02	0.95 \pm 0.04	0.95 \pm 0.03
Sucrose	342	0.98 \pm 0.02	1.00 \pm 0.06	1.19 \pm 0.12	1.20 \pm 0.15

*The extraction fluxes at pH 8.5 + 100 mM CaCl₂ were not significantly different, for any solute, from those at pH 6.3 + 30 mM NaCl.

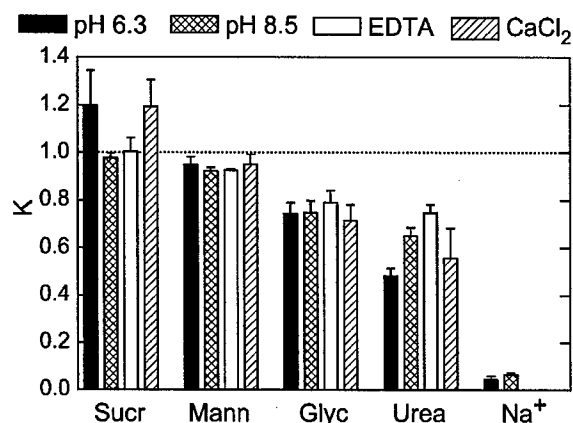


FIGURE 2 Values of the calibration constant K (mean \pm SD; $n = 4$) for each internal standard candidate at each set of experimental conditions studied (see Table 3).

$$K = \frac{J_{\text{Glu}}}{J_{\text{IS}}} = \frac{(J_{\text{EO,Glu}} + J_{\text{p,Glu}})}{(J_{\text{EO,IS}} + J_{\text{p,IS}})} \quad (8)$$

where J_{EO} and J_{p} refer to the electroosmotic and passive fluxes during iontophoresis of the solutes concerned: i.e., glucose (*Glu*) and the internal standard (*IS*). It follows, therefore, that if the J_{EO} are identical, K will not equal 1 if the passive contributions for glucose and the internal standard are different, and if they make up a measurable component of the experimentally measured fluxes.

Although this issue is of mechanistic interest, and relevant to previous work that has focused on the relative contributions of electromigration and electroosmosis to the iontophoresis of cationic drugs (Guy et al., 2000; Yoshida and Roberts, 1993), it must nevertheless be emphasized that this phenomenon elicited only a modest (no more than two-fold) effect on the absolute value of the calibration constant K . Of greater practical importance is whether, for any particular candidate internal standard, the value of K remains fixed despite changes in the skin's permselectivity. Such was clearly not the case when Na^+ was examined for this role in vivo (Sieg et al., 2004). For mannitol and glycerol, deviations of K were small. However, these solutes are either present physiologically at levels too low (mannitol, $\sim 34 \pm 18 \mu\text{M}$ (Lentner, 1984)) or are subject to significant systemic variation (unbound glycerol, $\sim 120 \pm 65 \mu\text{M}$ (Lentner, 1984)) to be useful internal standards for glucose monitoring in vivo. Sucrose and urea showed slightly greater deviations in K . Again, sucrose (plasma concentration $\sim 1.8 \pm 1.2 \mu\text{M}$ (Lentner, 1984)) is not a practical option for the same reason as mannitol. Urea outperformed Na^+ , both in terms of absolute divergence of the observed K , and in terms of variability. The fact that K for urea is >10 times the corresponding Na^+ value confirms that its extraction is primarily achieved by the same mechanism as glucose. In addition, blood concentrations of urea are relatively stable, sufficiently high (4–8 mM (Bankir and

Trinh-Trang-Tan, 2000)) and in rapid equilibrium with those in the extracellular fluid (the tissue compartment sampled by reverse iontophoresis) to offer a pragmatic option for a useful internal standard (Rosdahl et al., 1998). Finally, it is important to point out that the variability observed (coefficients of variation typically $<10\%$) in the values of K obtained with urea as an internal standard would be acceptable in clinical practice. In a recent study, in which lithium serum levels in bipolar patients were successfully predicted using sodium as an internal standard, the variability in K was on the order of no more than 7% (Leboulanger et al., 2004c).

Of course, it remains to be seen whether the conclusions from this work are directly applicable in vivo, in human subjects. It should be recalled that the skin model used, although considered extremely useful and faithful, was from pig rather than man. In addition, the subdermal milieu in vivo is much more complex than that employed in the in vitro reported here. Equally, although it has been possible to deliberately modify skin permselectivity in these experiments by modulating the cathodal formulation, there are undoubtedly other physiological and/or environmental factors that can also play a role in the real world and these issues can only be elucidated and examined in vivo (for example, although the intraindividual variation in the blood concentration of urea over a 24-h period is quite small, intersubject variability is greater, which means that it may be necessary to verify periodically a "personal" K for each diabetic (Bankir and Trinh-Trang-Tan, 2000)). Such is clearly the next logical step in this research.

This work was supported by the United States National Institutes of Health (EB 001420) and United States Army Medical Research Acquisition Activity grant DAMD17-02-1-0712 (Fort Detrick, MD). The information presented does not necessarily reflect the position or the policy of the United States government, and no official endorsement should be inferred.

REFERENCES

- Bankir, L., and M. M. Trinh-Trang-Tan. 2000. Urea and the kidney. In *The Kidney*. B. M. Brenner, editor. W. B. Saunders, Philadelphia, PA. 637–679.
- Burnette, R. R., and B. Ongpipattanakul. 1987. Characterization of the permselective properties of excised human skin during iontophoresis. *J. Pharm. Sci.* 76:765–773.
- Delgado-Charro, M. B., and R. H. Guy. 2003. Transdermal reverse iontophoresis of valproate: a non-invasive method for therapeutic drug monitoring. *Pharm. Res.* 20:1508–1513.
- Glikfeld, P., R. S. Hinz, and R. H. Guy. 1989. Noninvasive sampling of biological fluids by iontophoresis. *Pharm. Res.* 6:988–990.
- Guy, R. H., Y. N. Kalia, M. B. Delgado-Charro, V. Merino, A. Lopez, and D. Marro. 2000. Iontophoresis: electropulsion and electroosmosis. *J. Control. Release.* 64:129–132.
- Kalia, Y. N., A. Naik, J. Garrison, and R. H. Guy. 2004. Iontophoretic drug delivery. *Adv. Drug Deliv. Rev.* 56:619–658.
- Kim, A., P. G. Green, G. Rao, and R. H. Guy. 1993. Convective solvent flow across the skin during iontophoresis. *Pharm. Res.* 10:1315–1320.
- Leboulanger, B., R. H. Guy, and M. B. Delgado-Charro. 2004a. Reverse iontophoresis for non-invasive transdermal monitoring. *Physiol. Meas.* 25:R35–R50.

- Leboulanger, B., R. H. Guy, and M. B. Delgado-Charro. 2004b. Non-invasive monitoring of phenytoin by reverse iontophoresis. *Eur. J. Pharm. Sci.* 22:427–433.
- Leboulanger, B., J. M. Aubry, G. Bondolfi, R. H. Guy, and M. B. Delgado-Charro. 2004c. Non-invasive lithium monitoring by reverse iontophoresis in vivo. 10.1373/*Clin. Chem.* 2004.034249.
- Lentner, C. 1984. Geigy Scientific Tables, Vol. 3. Ciba-Geigy, Basel, Switzerland.
- Marro, D., R. H. Guy, and M. B. Delgado-Charro. 2001. Characterization of the iontophoretic permselectivity properties of human and pig skin. *J. Control. Release.* 70:213–217.
- Phipps, J. B., and J. R. Gyory. 1992. Transdermal ion migration. *Adv. Drug Deliv. Rev.* 9:137–176.
- Pikal, M. J. 1992. The role of electroosmotic flow in transdermal iontophoresis. *Adv. Drug Deliv. Rev.* 9:201–237.
- Potts, R. O., J. A. Tamada, and M. J. Tierney. 2002. Glucose monitoring by reverse iontophoresis. *Diabetes Metab. Res. Rev.* 18:S49–S53.
- Rao, G., R. H. Guy, P. Glikfeld, W. R. LaCourse, L. Leung, J. A. Tamada, R. O. Potts, and N. T. Azimi. 1995. Reverse iontophoresis: noninvasive glucose monitoring in vivo in humans. *Pharm. Res.* 12:1869–1873.
- Rosdahl, H., K. Hamrin, U. Ungerstedt, and J. Henrikson. 1998. Metabolite levels in human skeletal muscle and adipose tissue studied with microdialysis at low perfusion flow. *Am. J. Physiol.* 274:E939–E945.
- Ruddy, S. B., and B. W. Hadzija. 1992. Iontophoretic permeability of polyethylene glycols through hairless rat skin: application of hydrodynamic theory for hindered transport through liquid-filled pores. *Drug Des. Discov.* 8:207–224.
- Santi, P., and R. H. Guy. 1996a. Reverse iontophoresis—parameters determining electroosmotic flow: I. pH and ionic strength. *J. Control. Release.* 38:159–165.
- Santi, P., and R. H. Guy. 1996b. Reverse iontophoresis—parameters determining electroosmotic flow: II. Electrode chamber formulation. *J. Control. Release.* 42:29–36.
- Sieg, A., R. H. Guy, and M. B. Delgado-Charro. 2003. Reverse iontophoresis for noninvasive glucose monitoring: the internal standard concept. *J. Pharm. Sci.* 92:2295–2302.
- Sieg, A., R. H. Guy, and M. B. Delgado-Charro. 2004. Noninvasive glucose monitoring by reverse iontophoresis in vivo: application of the internal standard concept. *Clin. Chem.* 50:1383–1390.
- Tamada, J. A., N. J. V. Bohannon, and R. O. Potts. 1995. Measurement of glucose in diabetics subjects using non invasive transdermal extraction. *Nat. Med.* 1:1198–1201.
- Tierney, M. J., J. A. Tamada, R. O. Potts, L. Jovanovic, S. Garg, and Cygnus Research Team. 2001. Clinical evaluation of the GlucoWatch biographer: a continual, non-invasive glucose monitor for patients with diabetes. *Biosens. Bioelectron.* 16:621–629.
- Yoshida, N. H., and M. S. Roberts. 1993. Solute molecular size and transdermal iontophoresis across excised human skin. *J. Control. Release.* 25:177–195.

Simultaneous Extraction of Urea and Glucose by Reverse Iontophoresis *in Vivo*

Anke Sieg,^{1,2} Richard H. Guy,^{1,3} and
M. Begoña Delgado-Charro^{1,3,4}

Received April 27, 2004; accepted June 21, 2004

Purpose. Reverse iontophoresis extracts glucose across the skin in the GlucoWatch Biographer, a device to monitor glycemia in diabetes. However, the device must first be calibrated with an invasive "finger-stick" and this has been perceived as a disadvantage. Here, urea, a neutral "internal standard" is extracted simultaneously in an attempt to render the technique completely non-invasive.

Methods. In a 5-h experiment in human volunteers, reverse iontophoretic fluxes of glucose and urea (J_{glu} and J_{urea} , respectively) were measured periodically and correlated with the corresponding blood levels. In the case of glucose, a finger-tip blood sample was taken at the beginning of each collection interval; for urea, three blood samples were assayed: one before, one during, and one at the end of iontophoresis.

Results. The ratio $J_{\text{glu}}/J_{\text{urea}}$ divided by the ratio of the systemic concentrations ($C_{\text{glu}}/C_{\text{urea}}$) yielded an extraction coefficient (K) that could be compared between subjects. Though J_{glucose} tracked C_{glu} faithfully when the volunteers were challenged with an oral glucose load, J_{urea} remained quite stable reflecting the fact that C_{urea} did not change appreciably during the experiment. However, whereas the variability (expressed as the coefficient of variation) in the normalized extraction flux of urea ($J_{\text{urea}}/C_{\text{urea}}$) was on the order of 25%, that for glucose was greater (>45%), with the result that the values of K (0.45 ± 0.25) were less constant than anticipated.

Conclusions. Although urea performed quite reasonably as an internal standard, in that its extraction flux and systemic concentration both remained quite constant, the normalized, transdermal, iontophoretic flux of glucose showed interindividual variability due to mechanisms that were not entirely governed by electrotransport. That is, despite good qualitative tracking to blood levels, there appear to be other (biochemical, metabolic, contamination?) factors that impact upon the quantitative results obtained.

KEY WORDS: electroosmosis; iontophoresis; non-invasive glucose monitoring; skin; transdermal.

INTRODUCTION

The GlucoWatch Biographer uses reverse iontophoresis to non-invasively extract glucose across the skin (1). It allows glycemia in diabetics to be monitored over the course of a day. The iontophoretic extraction is based on the application of a low electric current, which drives the migration of ions across the skin. This biologic membrane, under physiologic

conditions, is negatively charged and, as a result, permselective to cations. The preferential passage of counter-ions across the skin induces electroosmosis, a convective solvent flow, which carries small neutral molecules such as glucose toward the skin surface. The electroosmotic flux of a solute (J_{EO}) is proportional to the potential gradient established by the electric field ($-d\Phi/dx$) and the subdermal concentration of the analyte C_i (2):

$$J_{\text{EO}} = L_{\text{VE}} \cdot \frac{-d\Phi}{dx} \cdot C_i \quad (1)$$

where the electroosmotic flow coefficient L_{VE} describes the direction and the magnitude of the volume flow. In addition to convective flow, passive diffusion may additionally contribute to the total transport across the skin. For a neutral species, the total flux J_i ($\text{nmol} \cdot \text{h}^{-1}$) can therefore be expressed as the sum of the electroosmotic (J_{EO}) and passive diffusion contributions (J_p):

$$J_i = J_{\text{EO}} + A \cdot J_p \quad (2)$$

where A (cm^2) is the surface area of the skin across which transport is occurring. Note that J_p is typically expressed, for example, in units of $\text{nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ and the total amount transported across a membrane by this mechanism depends directly on the available diffusion area (A). Further, it is generally accepted that J_p will be a function of the size of the diffusing molecule (3). In contrast, at constant current, J_{EO} is governed by the charge on, and hence the permselectivity of, the skin and appears to be area-independent (4). Additionally, J_{EO} is insensitive, or at least much less sensitive, to the size of the convected species (2). The magnitude of convective flow may be an important factor behind the variability in iontophoretic fluxes of neutral solutes.

Such inter- (and even intra-) individual differences mean that glucose monitoring with the GlucoWatch requires calibration against a conventional blood sample at each use. The invasive calibration procedure (a classic "finger-stick" to obtain a blood sample) has been perceived as a disadvantage, despite the fact that the device provides highly valuable information about the blood glucose profile over time and allows hypoglycaemia to be anticipated (5).

The goal of the work presented here is to avoid the conventional calibration step via the use of an "internal standard." This approach involves the extraction and subsequent analysis of two species simultaneously, the analyte of interest (glucose) whose concentration varies as a function of time, and the internal standard (IS), an endogenous molecule of known and essentially fixed physiologic levels (6). If the iontophoretic transport of glucose is independent of that of the other, then their iontophoretic extraction fluxes (J_{glu} and J_{IS} , respectively) should obey the following relationship:

$$\frac{J_{\text{glu}}}{J_{\text{IS}}} = K \cdot \frac{C_{\text{glu}}}{C_{\text{IS}}} \quad (3)$$

where C_{glu} and C_{IS} are the blood concentrations of the two substances, and K is a proportionality constant (or extraction coefficient), which may be defined by the combination and rearrangement of Equations 2 and 3:

¹ School of Pharmacy, University of Geneva, 1211 Geneva 4, Switzerland.

² Procter & Gamble, Rusham Park Technical Centre, Egham, Surrey, TW20 9NW, United Kingdom.

³ Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, United Kingdom.

⁴ To whom correspondence should be addressed. (e-mail: B.Delgado-Charro@bath.ac.uk or r.h.guy@bath.ac.uk)

$$K = \frac{J_{\text{glu}}}{J_{\text{IS}}} \cdot \frac{C_{\text{IS}}}{C_{\text{glu}}} = \frac{(J_{\text{EO,glu}} + A \cdot J_{\text{p,glu}})}{(J_{\text{EO,IS}} + A \cdot J_{\text{p,IS}})} \cdot \frac{C_{\text{IS}}}{C_{\text{glu}}} \quad (4)$$

Initial work investigated this idea *in vitro* and *in vivo* using the sodium ion as the internal standard (6,7). Though encouraging results were obtained *in vitro*, significant inter- and intra-individual variation in *K* was observed for a subset of the study population *in vivo*. This variability was due to fluctuations in the extraction efficiency of glucose; the Na⁺ electrotransport remained remarkably constant as hypothesized. Further *in vitro* studies suggested that a small neutral molecule extracted by the same mechanism as glucose would better reflect modified electroosmotic transport (Sieg *et al.*, submitted), and urea was identified as a potentially interesting internal standard candidate for *in vivo* evaluation. This article presents the results from a preliminary evaluation of this idea.

MATERIALS AND METHODS

Study Population

Nondiabetic subjects (age range: 25 to 40 years; 1 male, 5 females), with no history of skin disease participated in the study. Informed consent was obtained, the study protocol having been approved by the internal review board of the University of Geneva in accord with the principles outlined in the Declaration of Helsinki. Five subjects participated on a single occasion, while 1 volunteer participated twice.

Chemicals

Tris base (α,α,α -Tris-(hydroxymethyl)-methylamine), sodium chloride, D-glucose, hydrochloric acid, sodium hydroxide, diacetylmonoxime, thiosemicarbazid, sulfuric acid, and iron (III) chloride were purchased from Sigma-Aldrich (St. Quentin Fallavier, France) and were at least analytical grade. Deionized water (resistivity > 18.2 Mohm/cm²) was used to prepare all solutions.

Iontophoresis

Two cylindrical glass cells (diameter 1.6 cm, extraction surface 2 cm²), separated by a distance of 7 cm, were fixed with foam tape (3M Health Care, St. Paul, MN, USA) on the subject's ventral forearm. The anodal chamber was filled with 1.2 ml of 10 mM Tris-buffer at pH 8.5 containing 100 mM NaCl; the cathodal chamber contained the same volume of 10 mM Tris buffer alone. The slightly alkaline pH was used to maximize electroosmotic flow, a strategy already adopted in the GlucoWatch (8). Custom-made Ag/AgCl electrodes were inserted into the solutions and fixed 3–4 mm above the skin surface to ensure that no physical contact occurred. Direct current (*I* = 0.6 mA, current density = 0.3 mA/cm²) was passed for a total of 5 h and was controlled by a Phoresor II Auto (Iomed, Salt Lake City, UT, USA), an FDA-approved, constant current, iontophoretic power supply. Every 15 min post-initiation of the current, the entire cathodal solution was collected and replaced by 1.2 ml of fresh buffer. The samples were immediately frozen until analysis.

After 2.5 h of iontophoresis, the subjects ingested 75 g of glucose dissolved in 300 ml of water (Glucosum monohydricum Ph.Eur., Hänsler AG, Herisau, Switzerland), so as to provoke a significant change in blood sugar. From this point

onward, glycemia was measured before each subsequent 15-min collection interval using a conventional blood glucose monitor (Glucotrend 2, Roche Diagnostics, Mannheim, Germany). Blood urea levels were determined in 32 μ l of capillary blood from the finger-tip using a Reflotron Benchtop Analyser (Roche Diagnostics, Mannheim, Germany). Three spot measurements were made: immediately before starting iontophoresis, during the experiment at the time of the first blood glucose measurement, and directly after termination of iontophoresis.

Analytical Chemistry

Glucose was assayed by high-performance anion chromatography with pulsed amperometric detection on a gold electrode using an ion chromatograph (Dionex 600 system, Dionex, Sunnyvale, CA, USA) (7). Urea was determined by a colorimetric reaction using diacetylmonoxime reagent (9,10). This method had a linear response over the concentration range from 5 to 70 μ M; only citrulline provided a significant interference. The colored complex was allowed to form at room temperature, and absorption was measured at a wavelength of 520 nm (Perkin Elmer 554 Spectrophotometer, Perkin Elmer, Norwalk, CT, USA).

Data Analysis and Statistics

Iontophoretic fluxes were calculated from the amounts extracted in each collection period and plotted at the mid-time point of each interval; blood glucose concentrations were the values at the actual time of measurement. Blood urea levels for each sampling interval were estimated by linear extrapolation between the three spot measurements; for each volunteer, the regression ($r^2 \geq 0.97$) indicated that this approximation was reasonable. Typically, urea concentrations declined slightly over the 5-h experiment (0.25 ± 0.05 mM/h).

Normalized fluxes were determined by dividing the iontophoretic flux values of glucose and urea by the corresponding blood concentrations at each interval. Statistical differences were assessed by ANOVA, followed by a Newman-Keuls multiple comparison test, using GraphPad Prism 3.02 software (San Diego, CA, USA). Individual values of *K* were determined by linear regression of the extracted flux ratio vs. the ratio of the corresponding blood levels, for which significance was tested by ANOVA. Slopes of the regression line were then compared by analysis of covariance as described in Ref. 11. Unless otherwise stated, data are expressed as mean \pm SD.

RESULTS AND DISCUSSION

Local Effects of Iontophoresis

All subjects experienced a mild tingling sensation when the current was applied. The sensation was typically asymmetric, being more noticeable at the anode than at the cathode. Generally, the sensation diminished with time of current application and lasted no longer than 30 min. Iontophoresis caused the skin beneath the electrode chambers to become slightly erythematous, an effect which disappeared within 24 h of current termination. In addition, a few, small, punctuate lesions remained after the redness disappeared; these marks persisted for several days. These completely reversible effects

are similar to those that have been reported in the literature (12), and do not appear to impair skin barrier function.

Glucose Extraction

The iontophoretic transport of glucose, an uncharged, polar molecule, occurs by electroosmosis and is directly proportional to the subdermal concentration (2). However, a "warm-up" period is necessary to establish a pseudo-steady electroosmotic flow and to empty the glucose reservoir from the skin. The latter is due, at least in part, to local metabolism and is not reflective of glucose levels in the blood. The recommended "warm-up" period for the GlucoWatch G2 is 2 h, a period similarly adopted in this study. Figure 1 shows the reverse iontophoretic extraction profiles of glucose for 3 volunteers. Though the fluxes accurately tracked the systemic glucose concentration, the extraction efficiency varied between subjects. Table I illustrates this point and shows, for each experiment, the average extraction fluxes of glucose normalized by the corresponding blood concentrations (i.e., the values of $J_{\text{glu}}/C_{\text{glu}}$ which, in turn, are equal to the apparent electroosmotic flow rates). Although the intra-individual variability was modest (the mean coefficient of variation was 21%), there were considerable differences between individuals: for the seven experiments performed, $J_{\text{glu}}/C_{\text{glu}} = 8.5 \pm 3.2 \mu\text{l} \cdot \text{h}^{-1}$ (CV = 38%). While it is tempting, when considering the $J_{\text{glu}}/C_{\text{glu}}$ data in Table I, to suggest that two subpopulations might exist, there are insufficient data from this study to justify the premise. However, if the results are compared to the earlier *in vivo* investigation (7), then this trend is supported more clearly. Previously, it was found that, in 12 experiments, $J_{\text{glu}}/C_{\text{glu}} = 7.1 \pm 4.7 \mu\text{l} \cdot \text{h}^{-1}$, a result not significantly different from that found here. Again, intra-individual differences, with one exception, showed CVs of no more than 20%. But more striking is the observation shown in Fig. 2, wherein the combined data for normalized glucose flux from the earlier work and from this study appear to separate into a "high-extraction" group ($J_{\text{glu}}/C_{\text{glu}} > 8 \mu\text{l} \cdot \text{h}^{-1}$), and a group for which extraction is less (and, sometimes, much less) efficient.

Urea Extraction

Reverse iontophoresis has been used to monitor blood urea in patients with renal insufficiency (13), and a linear correlation between the iontophoretically extracted analyte and the corresponding blood levels was obtained. Extraction was performed, however, for only 5 min, during which time the samples almost certainly included significant levels of endogenous urea from the skin (14). This is supported by the normalized fluxes reported, which ranged from 190 to 610 $\mu\text{l} \cdot \text{h}^{-1} \cdot \text{cm}^{-2} \cdot \text{mA}^{-1}$, that is 15–20 times higher than those observed in the study described here ($17.4 \pm 4.7 \mu\text{l} \cdot \text{h}^{-1} \cdot \text{cm}^{-2} \cdot \text{mA}^{-1}$; CV = 27%).

Typical reverse iontophoretic urea fluxes are shown in the lower panels of Fig. 1(A–C). After the initially very high levels obtained, which were outside the calibration range of the assay (and therefore not shown), fluxes stabilized after ~60 min and generally followed the slight decrease in urea blood levels that occurred during the 5-h experiment. As for glucose, the small, neutral urea is principally extracted by electroosmosis. Passive diffusion, nevertheless, has been shown to contribute significantly to urea electrotransport

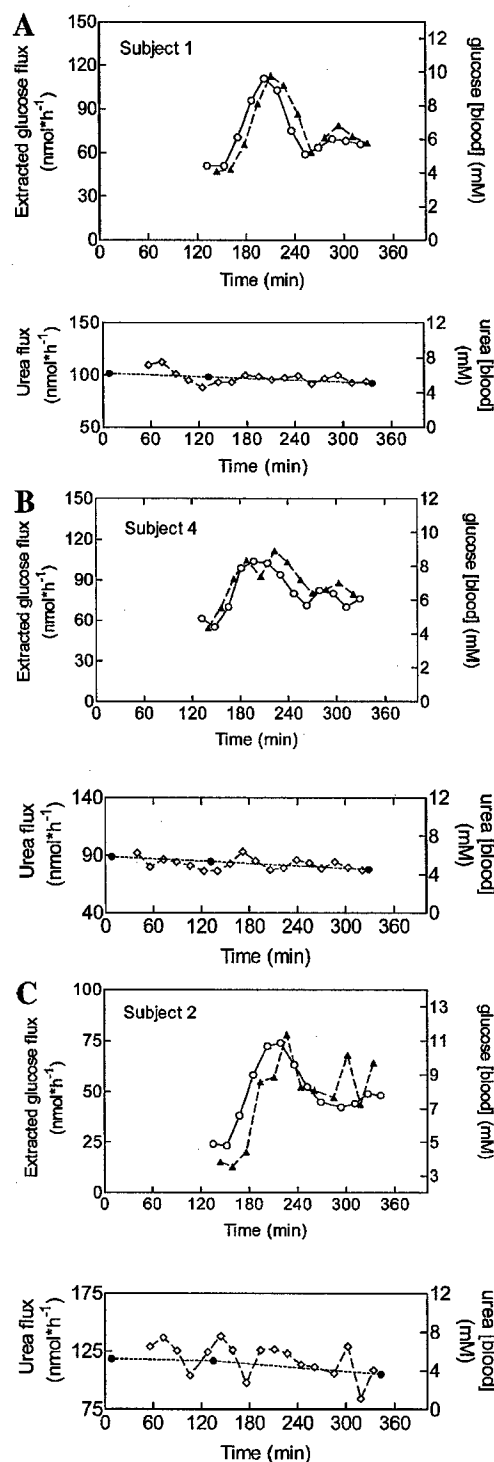


Fig. 1. Glucose (upper panels) and urea (lower panels) extraction profiles in 3 subjects. \circ — blood glucose (mM), \blacktriangle — glucose extraction flux ($\text{nmol} \cdot \text{h}^{-1}$), \bullet — blood urea (mM), \bullet — urea extraction flux ($\text{nmol} \cdot \text{h}^{-1}$)

(Sieg *et al.*, submitted), meaning that $J_{\text{urea}}/C_{\text{urea}}$ is greater than $J_{\text{glu}}/C_{\text{glu}}$, and that K should be less than 1 (see Eq. 4). Though the normalized urea fluxes ($20.7 \pm 5.6 \mu\text{l} \cdot \text{h}^{-1}$) also showed some inter-individual variation (Table I), the coefficient of variation (27%) was reasonable.

Table I. Iontophoretic Glucose and Urea Extraction Fluxes Normalized to the Corresponding Blood Levels^a

Subject	Blood glucose range (mM)	Blood urea range (mM)	Normalized glucose flux ($\mu\text{l} \cdot \text{h}^{-1}$)	Normalized urea flux ($\mu\text{l} \cdot \text{h}^{-1}$)	K \pm SD	r ²
1	4.4–9.6	5.0–6.3	11.8 \pm 0.9	17.6 \pm 0.8	0.67 \pm 0.04 ^c	0.97
2	4.8–10.9	3.6–5.3	5.8 \pm 2.1 ^b	26.1 \pm 3.6 ^b	0.34 \pm 0.05 ^d	0.79
3	4.6–10.3	3.8–5.4	11.1 \pm 3.1	16.5 \pm 2.2	0.86 \pm 0.11 ^c	0.88
4	4.4–8.3	4.4–5.8	13.6 \pm 1.5	16.5 \pm 1.1	0.63 \pm 0.09 ^c	0.83
5	2.6–10.9	5.3–7.1	5.8 \pm 1.5 ^b	15.9 \pm 2.1	0.44 \pm 0.05 ^d	0.89
6	3.7–7.6	5.0–6.2	4.6 \pm 1.0 ^b	28.5 \pm 2.5 ^b	0.16 \pm 0.04 ^d	0.62
3B	4.7–9.3	3.8–5.0	6.5 \pm 1.1 ^b	24.0 \pm 2.7 ^b	0.29 \pm 0.07 ^d	0.70

^a The extraction constant K was determined from linear regression as shown in Fig. 2, and the goodness of fit is expressed with r².

^b Significantly different from the fluxes of subjects 1–3 ($p < 0.001$).

^{c,d} Slopes are not significantly different from one another.

The apparent “bimodal” behavior with respect to glucose electrotransport was reinforced when the urea and glucose data were combined and analyzed by Eq. 3. Figure 3 plots the ratio $J_{\text{glu}}/J_{\text{urea}}$ against the corresponding ratio of the blood concentrations, and suggests quite persuasively that the subject population is divided into two subsets. Statistically, three of the seven regression slopes (i.e., the values of K given in Table I) were significantly different from the other four (0.72 ± 0.12 vs. 0.31 ± 0.12). Inspection of Table I reveals that this divergence originates primarily from the glucose data, rather than from those for urea, the CV for the normalized glucose fluxes being nearly twice that for urea. The effect is exacerbated by the fact that there is a weak inverse relationship between the measured values of $J_{\text{urea}}/C_{\text{urea}}$ and $J_{\text{glu}}/C_{\text{glu}}$.

Mechanism

Mechanistically, it was anticipated that the normalized glucose and urea fluxes would be closely correlated given that both molecules are moved across the skin by the same principal mechanism, i.e., electroosmosis. The higher passive permeation of urea was expected to result in a value of K less than 1 (Sieg *et al.*, submitted) but, given the relatively stable values of $J_{\text{urea}}/C_{\text{urea}}$, this cannot account for the divergent extraction coefficients observed. Equally, it seems unlikely that the explanation lies in the potential interference of citrulline in the urea assay. Although this substance is present

in the epidermis as a part of the skin’s “natural moisturizing factor” (15,16), this reservoir will have been substantially, if not completely, depleted by the 2 h of iontophoresis (17), which were performed before any measurements were made. Rather, it appears that there are possibly a diverse range of biochemical processes ongoing in living tissue (Fig. 4) that are more likely to impact upon the amount of glucose extracted (as, in fact, has been observed).

Iontophoretic extraction is thought to occur via two major pathways, namely the appendageal (sweat glands, hair follicles) and intercellular routes (18). In human skin, sweat glands and hair follicles have been identified as important low-resistance pathways (19); these structures are, at the same time, highly metabolically active (20), and glucose serves as a critical substrate for energy production in these and other skin cells (21). It is possible, therefore, that a fraction of the glucose being extracted through the skin by electroosmosis is “conscripted” for metabolism and this would lead to a significant reduction in the apparent normalized flux.

Additional physiologic factors that may interfere with the iontophoretic sampling of analytes are also highlighted in Fig. 4. As previously mentioned, skin reservoirs of glucose (12) and urea exist but have not been quantified, and the kinetics, with which these reservoirs can be emptied, have only been studied empirically. Thus, despite the 2-h pre-iontophoresis period, there is no certainty at the moment as to whether the skin depot(s) has (have) been completely depleted. Furthermore, the skin surface is another complicating factor. For example, perspiration may leave an elevated

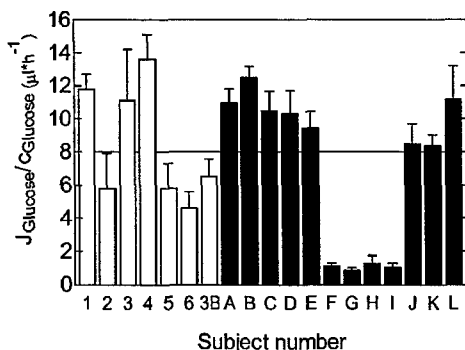


Fig. 2. Normalized reverse iontophoretic extraction fluxes of glucose *in vivo* (mean \pm SD) from this study (\square) and from a previous investigation (\blacksquare). The subjects showing more efficient extraction (some-what arbitrarily set at $8 \mu\text{l} \cdot \text{h}^{-1}$) have an average electroosmotic flow of $10.7 (\pm 1.6) \mu\text{l} \cdot \text{h}^{-1}$. For the less efficient group, the mean (\pm SD) convective flow is $3.3 (\pm 2.6) \mu\text{l} \cdot \text{h}^{-1}$.

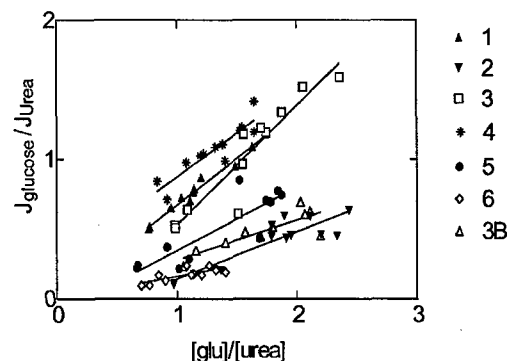


Fig. 3. Linear regressions of the ratio $J_{\text{glu}}/J_{\text{urea}}$ against the corresponding ratio of their blood concentrations ($C_{\text{glu}}/C_{\text{urea}}$). The slope of each line corresponds to the value of K, as defined in Eq. 3.

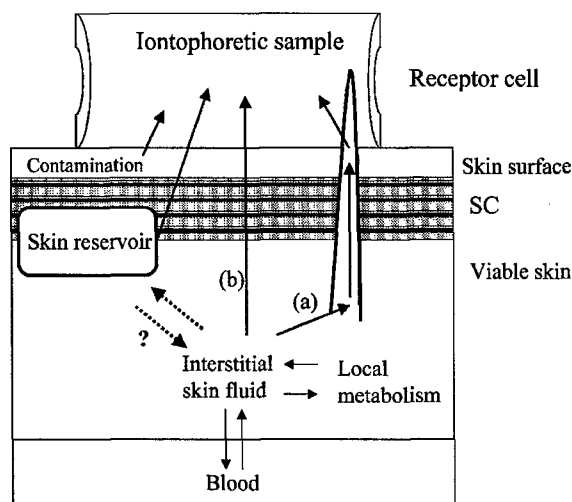


Fig. 4. Schematic diagram of the reverse iontophoresis sampling process. Iontophoresis extracts substances from the interstitial skin fluid (ISF) via the follicular (a) and the stratum corneum intercellular (b) pathways by electroosmosis, electromigration, and enhanced passive transport. Samples from capillary blood are used as references for concentration in the assumption that blood and ISF levels equilibrate relatively fast. ISF levels may also change as a consequence of local metabolism. Furthermore, a skin reservoir may exist due to local metabolism and distribution and/or SC differentiation. This depot, whose magnitude is not necessarily related to the ISF levels, will also be sampled by the application of iontophoresis. The skin surface may also contribute to the extracted sample via, for example, perspiration, microbial activity, or contamination from personal-care products. Finally, local metabolism and/or additional contamination may originate in skin appendages (including hair follicles and sweat glands).

amount of an analyte at the exit of the sweat gland (21,22); contamination of the skin surface by personal care products is also possible (23,24); and the skin microflora can be expected to play a role as well (25) in terms of glucose consumption and the apparent extraction rate of this analyte.

CONCLUSIONS

In summary, urea may prove to be a useful internal standard, in that its systemic concentration is quite stable and its reverse iontophoretic extraction flux proved to be quite constant (of course, this would probably not be the case for patients in severe renal failure who need to undergo regular dialysis). The electrotransport of glucose, however, continued to show inter-individual variability due to other factors that remain to be fully characterized. We would submit, therefore, that the real challenge for calibrating the reverse iontophoretic extraction of glucose via an internal standard is to understand and control the biochemical and metabolic processes occurring on and within the skin that have the potential to significantly perturb the outward movement of the analyte.

ACKNOWLEDGMENTS

This work was supported by the U.S. National Institutes of Health (EB 001420) and USAMRAA grant DAMD17-02-1-0712 (Fort Detrick, MD, USA). The information presented does not necessarily reflect the position or the policy of the U.S. Government, and no official endorsement should be inferred. We thank Dr. Russ Potts for many helpful discussions.

REFERENCES

1. M. J. Tierney, J. A. Tamada, R. O. Potts, L. Jovanovic, and S. Garg, and Cygnus Research Team. Clinical evaluation of the GlucoWatch Biographer: a continual, non-invasive glucose monitor for patients with diabetes. *Biosens. Bioelectron.* **16**:621–629 (2001).
2. M. J. Pikal. The role of electroosmotic flow in transdermal iontophoresis. *Adv. Drug Del. Rev.* **9**:201–237 (1992).
3. G. B. Kasting, R. L. Smith, and E. G. Cooper. Effect of lipid solubility and molecular size on percutaneous absorption. In B. Shroet and H. Schaefer (eds.), *Skin Pharmacokinetics*, Karger AG, Basel, 1987, pp. 138–153.
4. A. Lopez Castellanos, R. H. Guy, and M. B. Delgado-Charro. Effect of area on the iontophoretic transport of phenylalanine and propranolol across the skin. *Int. Symp. Control. Rel. Bioact. Mater* **24**:405–406 (1999).
5. K. R. Pitzer, S. Desai, T. Dunn, S. Edelmann, Y. Yakalakshmi, J. Kennedy, J. A. Tamada, and R. O. Potts. Detection of hypoglycemia with the GlucoWatch Biographer. *Diabetes Care* **24**:881–885 (2001).
6. A. Sieg, R. H. Guy, and M. B. Delgado-Charro. Reverse iontophoresis for noninvasive glucose monitoring: the internal standard concept. *J. Pharm. Sci.* **92**:2295–2302 (2003).
7. A. Sieg, R. H. Guy, and M. B. Delgado-Charro. Non-invasive glucose monitoring by reverse iontophoresis in vivo: application of the internal standard concept. *Clin. Chem.* **50**:1383–1390 (2004).
8. J. A. Tamada and R. O. Potts. Glucose monitoring using electroosmotic transdermal extraction. In B. Berner and S. M. Dinh (eds.), *Electronically Controlled Drug Delivery*, CRC Press, Boca Raton, 1998, pp. 161–173.
9. L. Goeyens, N. Kindermans, M. A. Yusuf, and M. Elskens. A room temperature procedure for the manual determination of urea in seawater. *Estuarine. Coastal and Shelf Science* **47**:415–418 (1998).
10. P. F. Mulvenna and G. Savidge. A modified manual method for the determination of urea in seawater using diacetylmonoxime reagent. *Estuarine. Coastal and Shelf Science* **34**:429–438 (1992).
11. J. H. Zar. Comparing simple linear regression equations. In J. H. Zar (ed.), *Biostatistical Analysis*, Prentice-Hall, Englewood Cliffs, NJ, 1984, pp. 292–327.
12. G. Rao, R. H. Guy, P. Glikfeld, W. R. LaCourse, L. Leung, J. A. Tamada, R. O. Potts, and N. T. Azimi. Reverse iontophoresis: non invasive glucose monitoring in vivo in humans. *Pharm. Res.* **12**:1869–1873 (1995).
13. I. T. Degim, S. Ilbasimis, R. Dundaroz, and Y. Oguz. Reverse iontophoresis: a non-invasive technique for measuring blood urea level. *Pediatr. Nephrol.* **18**:1032–1037 (2003).
14. K. Wellner and W. Wohlrab. Quantitative evaluation of urea in stratum corneum of human skin. *Arch. Dermatol. Res.* **285**:239–240 (1993).
15. A. V. Rawlings, I. R. Scott, C. R. Harding, and P. A. Bowser. Stratum corneum moisturization at the molecular level. *J. Invest. Dermatol.* **103**:731–740 (1994).
16. P. J. Caspers, G. W. Lucassen, E. A. Carter, H. A. Bruining, and G. J. Puppels. In vivo confocal raman microspectroscopy of the skin: noninvasive determination of molecular concentration profiles. *J. Invest. Dermatol.* **116**:434–442 (2001).
17. F. Jeanneret. Etude du liquide interstitiel cutané comme matrice alternative lors de suivi thérapeutique: couplage LC-MS pour l'analyse des acides aminés extraits par ionophorèse inverse in vitro et in vivo. Ph.D. Thesis, University of Geneva, 2004.
18. C. Cullander. What are the pathways of iontophoretic current flow through mammalian skin? *Adv. Drug Del. Rev.* **9**:119–135 (1992).
19. O. D. Uitto and H. S. White. Electroosmotic pore transport in human skin. *Pharm. Res.* **20**:646–652 (2003).

20. K. Sato. The physiology, pharmacology, and biochemistry of the eccrine sweat gland. *Rev. Physiol. Biochem. Pharmacol.* **79**:51–131 (1977).
21. R. K. Freinkel. Carbohydrate metabolism of epidermis. In L. A. Goldsmith (ed.), *Physiology, Biochemistry, and Molecular Biology of the Skin*, Oxford University Press, New York, 1993, pp. 452–461.
22. S. W. Brusilow. The permeability of the sweat gland to non-electrolytes. *Bibl. Paed.* **86**:32–40 (1966).
23. E. Proksch. Urea in dermatology. *Deut. med. Wochenschr.* **119**: 1126–1130 (1994).
24. H. Pratzel and K. Geiger. Zur Biochemie der freien Aminosäuren im Stratum Corneum menschlicher Epidermis I. Die Argininase-Reaktion. *Arch. Derm. Res.* **259**:151–156 (1977).
25. R. A. Bojar and K. T. Holland. Review: the human cutaneous microflora and factors controlling colonisation. *World J. Microb. Biot.* **18**:889–903 (2002).

Xiaoxia Bai
 Christophe Roussel
 Henrik Jensen
 Hubert H. Girault

Laboratoire d'Electrochimie
 Physique et Analytique,
 Institut de Chimie Moléculaire et
 Biologique, Ecole Polytechnique
 Fédérale de Lausanne,
 Lausanne, Switzerland

Polyelectrolyte-modified short microchannel for cation separation

Three alkali cations, potassium, sodium, and lithium, have been separated within 15 s in a 1 cm long polymer microchip. The separation microchannel is modified by a polycation, poly(allylammonium chloride), which makes the channel surfaces positively charged leading to a reversed electroosmotic flow (EOF) when compared to bare channels. Due to the decreased apparent mobility of the cations, the separation resolution is improved allowing the use of shorter channels.

Keywords: Capillary electrophoresis / Cation separation / Microchannel / Miniaturization / Polyelectrolyte / Surface modification
 DOI 10.1002/elps.200305771

1 Introduction

Micrototal analysis systems (μ TAS) allow fast and efficient chemical separation within small volumes. This is the main reason why they have attracted so much interest in recent years [1]. For pumping, electroosmotic flow (EOF) is usually the method of choice as it is rather easy to implement experimentally compared to pressure-driven flow [2–5]. However, the main weakness of EOF is the stability of the flow rate, and perhaps this is why capillary electrophoresis (CE) as a separation technique is not always as reproducible as required. To stabilize the EOF, it has been proposed to control the pH of the eluent, or to fix the zeta potential of the capillary either by chemical modification or by adsorption. In the latter case, adsorption of polyelectrolytes is rather an easy route to achieve this goal [6–10]. Except for modulating the EOF, these adsorption methods, both covalent and noncovalent, have also been used to prevent nonspecific adsorption, or as a means to attach active molecules (such as antibodies) to the surface. In the present paper, we demonstrate that polyelectrolyte adsorption can be used both to obtain a stable EOF as well as to improve the separation efficiency.

The separation of small ions is routinely carried out by traditional CE in silica capillaries [11–19], the ions being typically separated in minutes when the capillary length ranges from 10 to 80 cm. Analysis of small inorganic/organic charged species has also been performed on microchip platforms by capillary zone electrophoresis.

Correspondence: Professor Hubert Girault, Laboratoire d'Electrochimie Physique et Analytique, Institut de Chimie Moléculaire et Biologique, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland
E-mail: hubert.girault@epfl.ch
Fax: +41-21-693-3667

Abbreviations: His, L-histidine; HQ, hydroquinone; PAA-HCl, poly(allylamine hydrochloride) PE, polyethylene; PET, polyethylene terephthalate; SHE, standard hydrogen electrode

For example, separation of potassium, lithium, and sodium [20] was observed on a 8.4 cm long glass microchip within 1 min. Calcium and magnesium have also been separated within 15 s in a 2.5 cm long quartz microchip [21]. The separation of ammonium, methylammonium, and sodium has been obtained within 40 s in a 4 cm long polymer chip by using a movable contactless-conductivity detector [22].

Fast anion separation has been obtained using the co-electroosmotic separation mode on adding a cationic surfactant to the electrolyte that reverses the EOF, so that the anionic solutes electrophoretically migrate in the same direction as EOF [23–25]. Here, the same methodology is applied to generate a counter-electroosmotic mode of cation separation in order to decrease the length of the capillary. The length, as one of the size parameters, is an important aspect of microchip design where patterning density is a major issue. The present work describes a capillary electrophoresis analysis of Li^+ , Na^+ , and K^+ on a polymer chip using a polyelectrolyte coating of the micro channel surface with the objectives of performing a separation in 15 s within a 1 cm long separation channel. The long-term objective of this work is to design a lithium sensor for EOF reversed electrophoresis of extracted subcutaneous fluid where sample volume is a major issue.

2 Materials and methods

2.1 Chemicals

Polyethylene terephthalate (PET) sheets (100 μm thick Melinex) were purchased from Dupont (Geneva, Switzerland). 2-(*N*-Morpholino)ethanesulfonic acid (MES), L-histidine (His), poly(allylamine hydrochloride) (PAA-HCl) and hydroquinone (HQ) were obtained from Sigma (St. Louis, MO, USA). The standard ionic solutions with a concentra-

tion of 0.1 M were from Fluka (Buchs, Switzerland). Milli-Q water (Millipore, Bedford, MA, USA) was used to prepare all solutions. Freshly prepared solutions were used for the experiments.

2.2 Microchip fabrication

The fabrication of microchip has been previously described [26]. Briefly, the PET sheet is photoablated by a UV excimer laser (Argon Fluor Excimer Laser at 193 nm; Lambda Physik LPX 2051, Göttingen, Germany). The typical channel obtained has a trapezoidal cross-section shape. Carbon ink is pasted into a photoablated microchannel (with a depth of 20 μm and a width of 100 μm) and dried at 80°C for 30 min to form a conductive track. A photoresist solution (Shipley Europe, Herald Way, Coventry, UK) is used to seal and protect the carbon track, by spin-coating over the carbon track and thermally treating at 90°C for 1 h. In a second step, the main separation channel (corresponding to the horizontal channel shown in Fig. 1a) is photoablated perpendicularly to the carbon track so as to expose two face-to-face microelectrodes on the opposite vertical walls of the separation channel (Fig. 1b). The separation channel is usually about 40 μm deep and 50 μm wide at the top. Two side channels (corresponding to the two vertical side channels shown in Fig. 1a) are photoablated for injection purposes, which feature a so-called double-T design with a typical center-to-center distance of 100 μm . The microchip obtained is thermally laminated by a polyethylene/polyethylene terephthalate (PE/PET) layer (35 μm thick; Morane, Oxon, UK) at 135°C and 2 bar. The obtained microchip is similar

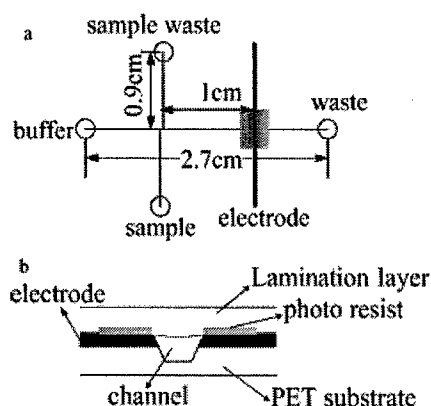


Figure 1. Scheme (a) of the top view of microchip and (b) the cross-section at the detector position. Two 0.9 cm long side channels (vertical) feature a double-T for injection purpose, two face-to-face microelectrodes are perpendicularly located at the end of the main separation channel.

to that shown in Fig. 1a (the top view) and Fig. 1b (the cross-section) except the effective separation length (from the double-T to the detection electrode) which may be different depending on the chip surface chemistry. The chip shown in Fig. 1a is indeed a polycation-coated microchip that has a shorter effective length of 1 cm.

2.3 CE on microchip

The microelectrodes are connected to a Metrohm conductivity meter (732 conductivity detector; Metrohm, Herisau, Switzerland) controlled by a PC through an interface (762 interface, Metrohm) [27]. A floating battery with 5 kV output and a locally made switching box are controlled by a PC through a Labview VI program (National Instruments, Austin, TX, USA). An electrokinetic floating injection is used to inject the sample: a positive potential of 500 V is applied for 20 s between the sample and sample waste reservoirs to fill the sample segment of the channel between these two reservoirs while the buffer and waste reservoirs are kept floating (no pinch). A positive potential of 800 V or 2 kV is applied between the buffer reservoir and the waste reservoir during a typical separation time of 50 s whilst the sample reservoirs are floating. Special attention has been given to the volume of the solutions in all the reservoirs. It is observed that when the solution volume in the buffer reservoir is smaller than that of other reservoirs, the sample plug in the double-T section migrates to the buffer reservoir during injection due to the effect of the hydrodynamic pressure, and a sample leakage from the sample channel to the main channel is observed after injection. A solution of 20 mM MES with 20 mM His (pH at 6) is used as background electrolyte (BGE) to separate cations [18]. HQ is added to the BGE to avoid the bubble generation due to an electrical cross-talk between the AC detection field (between the two face-to-face carbon electrodes) and the main DC high voltage. According to the redox potential of HQ (0.699 V vs. standard hydrogen electrode (SHE)), it can be easily oxidized into benzoquinone before water (1.23 V vs. SHE) to prevent the electrolysis of water that leads the formation of hydrogen (at cathode) and oxygen (at anode). The EOF measurement follows Huang's current monitoring method [28]. The principle of the measurement is to follow the conductivity change in a channel by monitoring the current at a constant voltage. When a solution of slightly higher conductivity is pumped (using a high voltage) inside a microchannel containing the same solution with lower conductivity, an increase and finally a stabilization of the current is observed, which indicates the time required by the solution to run through the channel. Measurement is performed with 4 mM and 20 mM MES/His

solutions to obtain an obvious current change, and a typical electrical field of 330 V/cm (by Spellman CZE 1000R Power Supply; Hauppauge, NY, USA) is applied to the microchannel.

2.4 Surface modification of microchannel

The surface modification of the PET channel by PAA-HCl is similar to that in a previously published work [29]. It was reported that the PAA-HCl absorbs on PET at low pH but reacts by amidation at high pH as a free base to form a covalently attached PAA-HCl layer. Therefore, PET-NH₂ samples prepared at high pH (such as 11.5) contain physisorbed PAA-HCl as well as chemisorbed PAA-HCl. Briefly, after washing with distilled water, the microchannel is filled with a PAA-HCl solution of 0.02 M repeat unit (corresponding to the concentration of the monomer) at pH 11.5 (adjusted by addition of 0.1 M NaOH) for 1 h. The channel is washed again with distilled water before filling with HCl (pH 2) for 30 min. The channel finally needs to be cleaned by distilled water and kept in air when it is not in use. The formation of the PAA-HCl layer is confirmed by EOF measurements. The electroosmotic mobility of a bare PET channel was measured to be $3.15 \times 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$ and the electroosmotic mobility of a PAA-HCl coated PET channel was $-3.52 \times 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$. It shows that the magnitude of the electroosmotic mobility does not change much after surface coating but the flow direction is reversed. This layer is quite stable when the chip is kept in air, similar results can be reproduced after several days.

3 Results and discussion

A baseline separation of three cations, potassium, sodium, and lithium, is obtained in a bare PET microchip with an effective length of 4.5 cm (electric field of 308 V/cm), as shown in Fig. 2. The resolutions between potassium and sodium, sodium and lithium are 2.14 and 1.63, respectively. The theoretical plate number is calculated as 35 600/m for potassium ion.

The resolution of the separation is given by

$$R_s = \frac{t_2 - t_1}{\frac{1}{2}(w_1 + w_2)} \quad (1)$$

where t is the migration time and w is the baseline bandwidth of the signal, proportional to the difference of the migration time, Δt . This difference can be expressed in terms of the apparent mobility u

$$\Delta t = t_2 - t_1 = \frac{L}{E} \left(\frac{u_1 - u_2}{u_1 u_2} \right) \quad (2)$$

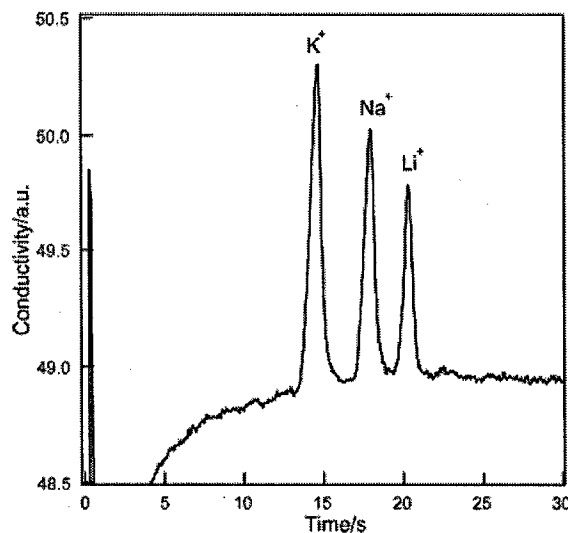


Figure 2. Electropherogram of ions. Channel dimension, 50 μm wide, 40 μm deep, and with 4.5 cm effective separation length; sample, 1 mM K⁺, Li⁺, and Na⁺, injected at 500 V for 25 s and separated at an electrical field of 308 V/cm; BGE, 20 mM MES/His, pH at 6.

where L is the effective length of the separation channel and E is the electrical field strength. The resolution is also related to the width of the peak, which can be represented by the variance σ and therefore to the apparent mobility,

$$w = 4\sigma = 4\sqrt{2Dt} = 4\sqrt{\frac{2DL}{E} \frac{1}{u}} \quad (3)$$

where D is the diffusion coefficient. Then, the separation resolution can be written as

$$R_s = \frac{u_1 - u_2}{u_2 \sqrt{u_1} + u_1 \sqrt{u_2}} \sqrt{\frac{L}{8DE}} \quad (4)$$

The apparent mobility is the sum of the electroosmotic mobility and the electrophoretic mobility. The sign of the former depends on the sign of the zeta potential. For a bare channel, the charges on the channel are negative, e.g., carboxy groups, the zeta potential is therefore negative and the electroosmotic mobility is positive, i.e., the EOF goes in the same direction as the electric field. The sign of the electrophoretic mobility depends on the sign of the ions, and is positive for the cations. By reversing the charges on the microchannel walls by adsorption of the polycation, the electroosmotic mobility becomes negative. The difference $u_1 - u_2$ in Eq. (2) is not altered by reversing the flow direction, but the apparent mobility u of the cations becomes smaller when the flow is reversed. Indeed, for a bare channel the electroosmotic and the electrophoretic mobilities add for cations, whereas in the modified channel the electroosmotic mobility is sub-

tracted from the electrophoretic one. As a consequence, the ratio $\frac{1}{(u_2\sqrt{u_1}+u_1\sqrt{u_2})^{-1}}$ is increased upon the modification of the channel. In other words, to obtain a similar separation with a fixed resolution, either the length of the channel could be decreased or the electrical field strength could be increased.

The following experiment confirms this approach. A microchip with a short effective separation length, 1 cm (as shown in Fig. 1a), is used to separate the same cations as mentioned before. The electropherograms obtained in this microchip before and after PAA-HCl coating are compared in Fig. 3. As it can be seen from Fig. 3a, although

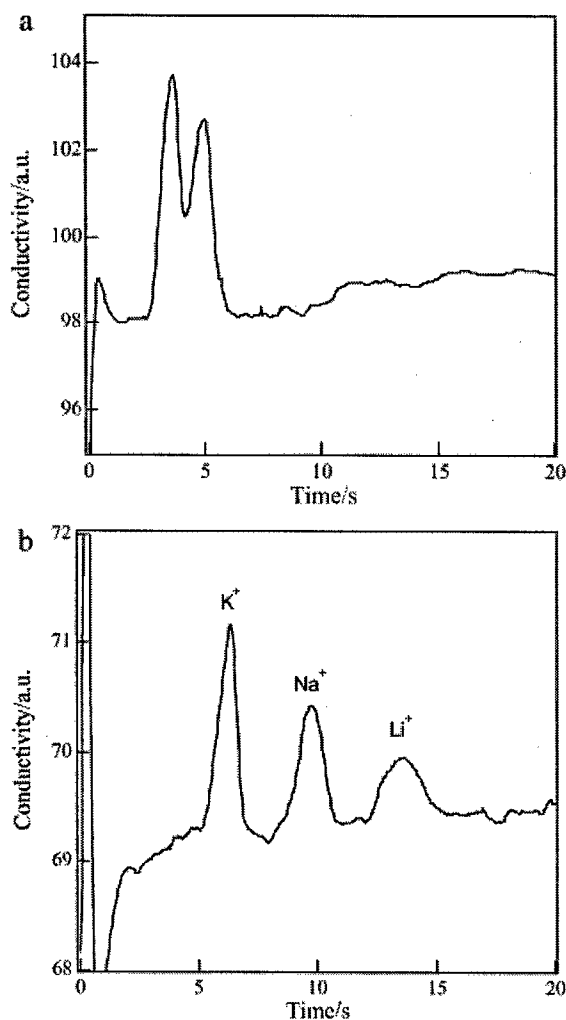


Figure 3. Comparison of the electropherograms obtained on a microchip (a) before and (b) after a PAA-HCl coated layer on the channel surface. Effective length, 1 cm; sample injection at 500 V for 20 s, separation at an electrical field of (a) 296 or (b) 741 V/cm; BGE, 20 mM MES/His+4 mM HQ, pH at 6. Other conditions as in Fig. 2.

each ion has not been identified respectively, the three ions cannot be separated in the bare PET microchannel when the electrical field is 296 V/cm, i.e., about the same electric field strength as in Fig. 2. It has to be pointed out that the other broadening effects, such as the detector region width and the injection plug width, may improve the detected signal. For instance, the separation can be accelerated by injecting a very small plug, and a confocal fluorescence detection focusing in the center of the channel can also improve the resolution of the separation [30]. However, those are out of the scope of the present work and therefore have not been evaluated here. According to Eq. (4), when the effective separation length is decreased by a certain factor, the electrical field should be decreased by the same factor in order to obtain a separation with the same resolution. For this microchannel with an effective length of 1 cm, the electrical field should be 68 V/cm to obtain a separation similar to that shown in Fig. 2. When the channel is coated with a PAA-HCl layer, a baseline separation is obtained even at an electrical field of 740.7 V/cm, (Fig. 3b). With this flow reversal method, a high electrical field, instead of a much lower electrical field as in the usual CE, can be applied when the effective length is highly decreased. The resolution between potassium and sodium is calculated to be 1.68 and 1.23 between sodium and lithium. The relatively small value of the resolution between sodium and lithium is mainly due to the broadening of the lithium signal. The theoretical plate number for potassium is 29 000/m. Compared to the separation shown in Fig. 2, both the resolution and the theoretical plate number are only slightly decreased. As shown in Eqs. (2) and (4), the migration time can be further decreased by increasing the applied electrical field, at the cost of reduced resolution (linear dependence vs. square root dependence). Those data show that the short channel coated with a PAA-HCl layer yields a similar electrophoresis efficiency for the small cations separation compared to the longer bare channel.

The presented experiments show a separation of cations for whose electrophoretic mobility is higher than the electroosmotic one. This method can also be applied to cations that have relatively small electrophoretic mobility. In this case, the electric field direction needs to be reversed for the cations to pass the detector and the apparent mobility is then negative.

In conclusion, we developed a surface modification method for polymer microchip to decrease the effective separation length of the chip whilst maintaining the resolution. This is important to microdevice design strategies as microfabrication techniques impose restriction on the

size of the devices. The present method therefore allows higher packing densities when fabricating CE, e.g., by plasma etching methods [31].

The authors wish to thank Metrohm (CH) and the Swiss Commission for Technology and Innovation (CTI: 4421.2) and Professor R. Guy (University of Geneva) and the US army medical research (DAXD17-02-1-0712) for financial support.

Received August 17, 2003

4 References

- [1] Reyes, D. R., Iossifidis, D., Auroux, P. A., Manz, A., *Anal. Chem.* 2002, 74, 2623–2636.
- [2] Dolnik, V., Liu, S. R., Jovanovich, S., *Electrophoresis* 2000, 21, 41–54.
- [3] Bousse, L., Cohen, C., Nikiforov, T., Chow, A., Kopf-Sill, A. R., Dubrow, R., Parce, J. W., *Ann. Rev. Biophys. Biomol. Struct.* 2000, 29, 155–181.
- [4] Bruin, G. J. M., *Electrophoresis* 2000, 21, 3931–3951.
- [5] Lacher, N. A., Garrison, K. E., Martin, R. S., Lunte, S. M., *Electrophoresis* 2001, 22, 2526–2536.
- [6] Graul, T. W., Schlenoff, J. B., *Anal. Chem.* 1999, 71, 4007–4013.
- [7] Liu, Y., Fanguy, J. C., Bledsoe, J. M., Henry, C. S., *Anal. Chem.* 2000, 72, 5939–5944.
- [8] Barker, S. L. R., Tarlov, M. J., Canavan, H., Hickman, J. J., Locascio, L. E., *Anal. Chem.* 2000, 72, 4899–4903.
- [9] Barker, S. L. R., Ross, D., Tarlov, M. J., Gaitan, M., Locascio, L. E., *Anal. Chem.* 2000, 72, 5925–5929.
- [10] Kamande, M. W., Kapnissi, C. P., Zhu, X. F., Akbay, C., Warner, I. M., *Electrophoresis* 2003, 24, 945–951.
- [11] Gross, L., Yeung, E. S., *Anal. Chem.* 1990, 62, 427–431.
- [12] Beck, W., Engelhardt, H., *Chromatographia* 1992, 33, 313–316.
- [13] Lin, T. I., Lee, Y. H., Chen, Y. C., *J. Chromatogr.* 1993, 654, 167–176.
- [14] Shi, Y., Fritz, J. S., *J. Chromatogr.* 1994, 671, 429–435.
- [15] Mayrhofer, K., Zemmann, A. J., Schnell, E., Bonn, G. K., *Anal. Chem.* 1999, 71, 3828–3833.
- [16] Francois, C., Morin, P., Dreux, M., *J. Chromatogr. A* 1995, 706, 535–553.
- [17] Soga, T., Ueno, Y., Naraoka, H., Matsuda, K., Tomita, M., Nishioka, T., *Anal. Chem.* 2002, 74, 6224–6229.
- [18] Kubáň, P., Kubáň, P., Kubáň, V., *Electrophoresis* 2002, 23, 3725–3734.
- [19] Tanyanyiwa, J., Hauser, P. C., *Electrophoresis* 2002, 23, 3781–3786.
- [20] Gardeniers, H., Mulder, M., Luttge, R., van den Berg, A., *MST-News* 2002, 4, 39–40.
- [21] Kutter, J. P., Ramsey, R. S., Jacobson, S. C., Ramsey, J. M., *J. Microcol. Sep.* 1998, 10, 313–319.
- [22] Wang, J., Chen, G., AMuck, A. J., *Anal. Chem.* 2003, 75, 4475–4479.
- [23] Zemmann, A., Nguyen, D. T., Bonn, G., *Electrophoresis* 1997, 18, 1142–1147.
- [24] Cugat, M. J., Borrull, F., Calull, M., *Chromatographia* 1999, 50, 229–234.
- [25] Cugat, M. J., Borrull, F., Calull, M., *Chromatographia* 1999, 49, 261–267.
- [26] Roberts, M. A., Rossier, J. S., Bercier, P., Girault, H. H., *Anal. Chem.* 1997, 69, 2035–2042.
- [27] Bai, X., Lee, H. J., Rossier, J. S., Reymond, F., Schafer, H., M., W., Girault, H. H., *Lab on a Chip* 2002, 2, 45–49.
- [28] Huang, X. H., Gordon, M. J., Zare, R. N., *Anal. Chem.* 1988, 60, 1837–1838.
- [29] Chen, W., McCarthy, T. J., *Macromolecules* 1997, 30, 78–86.
- [30] Bai, X. X., Josserand, J., Jensen, H., Rossier, J. S., Girault, H. H., *Anal. Chem.* 2002, 74, 6205–6215.
- [31] Rossier, J. S., Vollet, C., Carnal, A., Laguer, G., Gobry, V., Girault, H. H., Michel, P., Reymond, F., *Lab on a Chip* 2002, 2, 145–150.

Calibration-Free Glucose Monitoring Using Reverse Iontophoresis *In Vivo*

A. Sieg^{1,2}, R.H. Guy^{1,2}, M.B. Delgado-Charro^{1,2}

¹ School of Pharmacy, University of Geneva, 1211 Geneva 4, Switzerland

² Centre interuniversitaire de recherche et d'enseignement, Campus universitaire, 74160 Archamps, France
Anke.Sieg@pharm.unige.ch

SUMMARY:

A non-invasive, calibration-free method to extract glucose across the skin was developed. It comprises the simultaneous iontophoretic extraction and quantitation of the analyte glucose and an internal standard (the sodium ion) allowing glycemia to be determined without the need for a calibrating blood sample.

KEYWORDS:

iontophoresis, electro-osmosis

INTRODUCTION:

Frequent self-monitoring of glucose is essential for a safe and effective diabetes therapy. Conventional self-testing methods require a blood sample and have poor patient compliance. Consequently, there is considerable interest in providing non-invasive and continuous glucose monitoring technologies. The use of reverse iontophoresis is an example and has led to the development of the GlucoWatch[®] Biographer (Cygnus Inc., Redwood City, CA). The device monitors glucose continuously for up to 13 hours, recording six glucose readings per hour. However, before each use, it must be calibrated with a conventional blood sample to correlate the extracted glucose amounts with subdermal levels. This essential step has been perceived as a disadvantage.

The concept addressed here is that of an internal standard. As iontophoresis is non-specific, many ions and small uncharged species are moved across the skin when the current is applied. We monitor the extraction of two species simultaneously: (i) glucose, and (ii) Na⁺, the physiological concentration of which is known and essentially fixed. If the iontophoretic transport of glucose (G) and the "internal standard" (Na⁺) are independent of one another, then their fluxes (J) into collection devices on the skin surface should obey the relationship: $J_G/J_{Na} = K * [G]/[Na]$, where [G] and [Na] are the respective blood concentrations, and K is a constant.

EXPERIMENTAL METHODS:

In vitro

Dermatomed porcine ear skin was clamped into side-by-side diffusion cells (transport area = 0.78 cm²), with the stratum corneum side facing the

cathodal chamber. Constant current (0.5 mA/cm²) was applied for 6 hrs via Ag/AgCl electrodes. In a first study, mannitol was used as a model for glucose. Its concentration in the subdermal compartment was varied from 3 to 10 mM (and "spiked" with ¹⁴C-mannitol). The background electrolyte was NaCl, varied over its physiological range (125-145 mM), and 4 mM KCl in 25 mM Tris buffer at pH 7.4. Each time that the subdermal solution was changed (i.e., every 60 minutes), the entire contents of the cathodal chamber were withdrawn (for analysis of mannitol by liquid scintillation counting and Na⁺ by an ion-selective electrode) and replaced with fresh buffer. Secondly, the simultaneous reverse iontophoretic extraction of glucose and mannitol was studied. The subdermal solution always contained 10 mM sugar comprising symmetric mixtures of glucose ("spiked" with ³H) and mannitol in pH 7.4 Tris buffer containing 133 mM NaCl and 4 mM KCl. The cathodal chamber was sampled every hour over 6 hours for glucose, mannitol and Na⁺.

In vivo

Glass electrode chambers (2 cm² area) were fixed to the forearm of human volunteers. The anode held 10 mM Tris buffer (pH 8.5) + 100 mM NaCl; the cathode contained buffer alone. Constant current (0.6 mA) was passed for 5 hours. Every 15 minutes, the cathode solution was removed for analysis and then re-filled. The cycle was repeated over a total of 5 hours, during which the subjects received a 75g oral glucose load. Blood glucose concentrations were measured at the beginning and end of each iontophoretic sampling period using a conventional 'finger-stick' procedure. The cathode samples were analysed for glucose and Na⁺ by high-performance ion chromatography.

RESULTS AND DISCUSSION:

In vitro, the extracted mannitol flux reflected proportionally its subdermal concentration, whereas the sodium flux remained constant during the experiment (Figure 1). Equally, the simultaneous extraction of mannitol and glucose, which are neutral molecules, showed a linear relationship between their fluxes and their subdermal concentrations. Both sugars were extracted by electroosmosis with the same efficiency, showing no significantly different fluxes. Sodium ions, which are transported by electromigration, carried the major part of the current

($t_{Na^+} \sim 0.55$). The sodium extraction flux was independent of the subdermal Na^+ concentration over the physiological range.

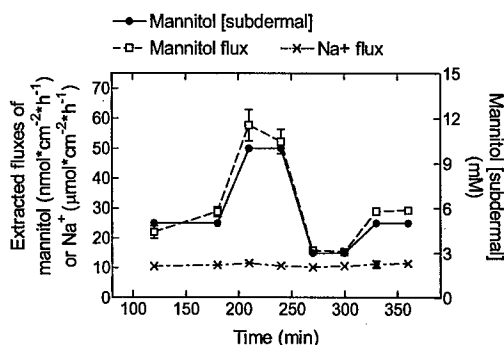


Figure 1: Extraction fluxes of mannitol and sodium *in vitro* ($n=4$)

The results from one of the volunteers, in whom appreciable changes in blood glucose were seen during the 5-hour experiment, are shown in Figure 2. It is noteworthy that (i) the extracted glucose flux tracks with excellent fidelity the changes in blood sugar, and (ii) the extracted Na^+ flux is very constant indeed. The linear correlations in Figure 3 confirm that the "internal standard concept" functions *in vivo* in man ($n = 5$). The extracted flux ratio of glucose relative to Na^+ varies linearly with the blood glucose concentration and with the ratio of the systemic concentrations of glucose to Na^+ ; that is, the data conform to the 'internal standard hypothesis':

$$J_G/J_{Na} = K * [G]/[Na]$$

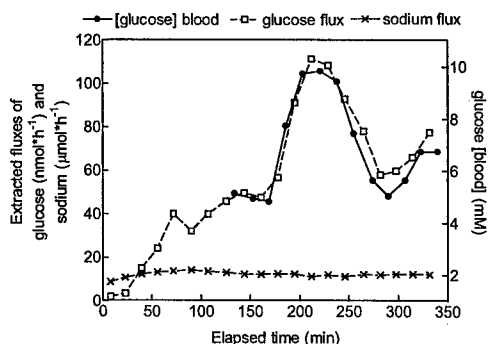


Figure 2: *In vivo* extraction of glucose and sodium (1 subject)

For these first volunteers studied, the "constant" (K) is really quite constant; the absolute value (0.12 ± 0.017) being nearly twice that observed *in vitro* (0.072 ± 0.011) suggesting a somewhat more efficient extraction of glucose in the *in vivo* situation. The relatively small values of K almost certainly reflect the different efficiencies of Na^+

electromigration and neutral molecule electroosmosis.

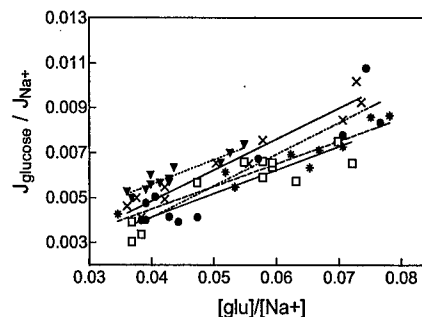


Figure 3: Extracted flux ratio as a function of the subdermal concentration ratio *in vivo* (5 subjects)

CONCLUSION:

The results presented here demonstrate the development of the reverse iontophoresis approach towards a truly non-invasive system that would not require calibration with a blood sample. This obvious advantage must be weighed against the need to identify and validate a suitable internal standard (that is, for glucose, would the ideal internal standard, in fact, be another uncharged compound, of similar physiological properties, whose systemic concentration, of course, is essentially invariant) and the additional analytical chemistry necessary for the practical quantification of two extracted substances.

REFERENCES:

1. Rao G et al., *Pharm Res* 10 (1993) 1751-1755
2. Tierney MJ et al., *Ann Med* 32 (2000) 632-641
3. Tamada JA et al., *Nature Med* 1 (1995) 1198-1201
4. Santi P, Guy RH, *J Control Rel* 38 (1996) 159-165
5. Burnette RR, Ongpipattanukul J, *J Pharm Sci* 76 (1987) 765-773

ACKNOWLEDGEMENTS

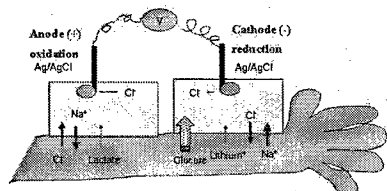
This work was supported by the "Programme commune de recherche en génie biomédical" of the EPFL and the Universities of Lausanne and Geneva, Switzerland, and by USAMRAA grant DAMD17-02-1-0712 (Fort Detrick, MD). The information presented does not necessarily reflect the position or the policy of the U.S. Government, and no official endorsement should be inferred.

Non-invasive Monitoring of Lactate by Reverse Iontophoresis

¹S. Nixon, ^{1,2}M.B. Delgado-Charro and ^{1,2}R.H. Guy

¹School of Pharmacy, University of Geneva, Switzerland & ²Dept. Pharmacy and Pharmacology, University of Bath, UK.

BACKGROUND



- o Iontophoresis enhances the transdermal transport of molecules via the application of a small electrical current (≤ 0.6 mA). This technique can be applied both to drug delivery and non-invasive monitoring (Fig.1) (1).
- o The Glucowatch Biographer® relies on reverse iontophoresis to measure glucose levels in diabetics (2).
- o The refinement of the procedure by the use of an internal standard allows lithium to be non-invasively monitored (Fig. 2) (3).

Figure 1

$$\frac{J_{Li}}{J_{Na}} = k \frac{C_{Li}}{C_{Na}} = \gamma C_{Li}$$

INTRODUCTION

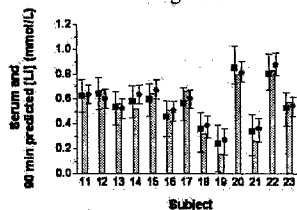


Figure 2

L-lactate is used as a marker in critically ill patients and as indicator of performance in sports training. The specific aims of this work, therefore, are:

- o To investigate, *in vitro* and *in vivo*, whether transdermal iontophoresis can be used to monitor lactate.
- o Is the reverse iontophoretic extraction flux of lactate ($J_{lactate}$) proportional to its subdermal concentration ($C_{lactate}$)?

IN VITRO EXPERIMENTS

- o Iontophoresis (0.4 mA & 5 h & Ag/AgCl electrodes) was performed on pig ear skin.
- o The subdermal solution contained L-lactate (0.5-4 mM) in a pH 7.4 buffer (25 mM Hepes, 133 mM NaCl). The electrode solutions were 50 mM NaCl. L-lactate was assayed enzymatically.

Results

1. Passive lactate flux was negligible compared to iontophoretic transport.
2. A lactate reservoir exists in excised pig skin (Fig.3)
3. Once the reservoir is emptied (~2 hours), lactate iontophoretic fluxes correlate well with the subdermal concentration. The value of the efficiency of extraction ($\gamma = J_{lactate}/C_{lactate}$) was $9.4 \pm 0.9 \mu\text{L/h}$ (Fig.4).

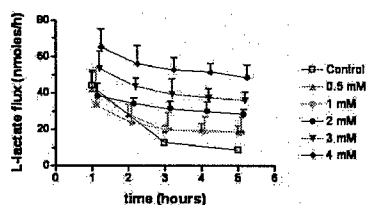


Figure 3

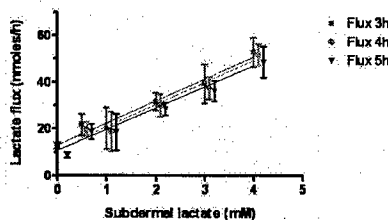


Figure 4

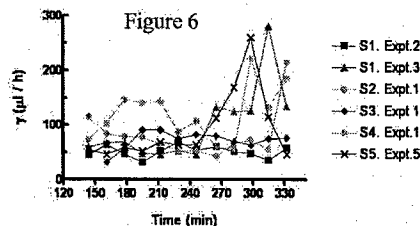
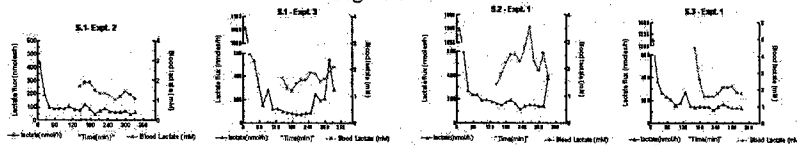
IN VIVO EXPERIMENTS

- o Iontophoresis (0.6 mA & 5 h & 0.3 mA/cm^2) was performed on 5 healthy volunteers. Subject 1 participated twice. The electrode solutions were 50 mM NaCl. The anodal solution was removed every 15 minutes. L-lactate was assayed by ionic chromatography.
- o Lactate blood levels were monitored simultaneously using a marketed device (Accusport®).

Results

1. A lactate reservoir exists in human skin which was emptied after ~1 hour iontophoresis.
2. Lactate was easily extracted (Fig.5). The efficiency of extraction (Fig. 6) was relatively constant for 3 subjects, resulting in values of $51.3 (\pm 11)$, $69.3 (\pm 20)$ and $71.9 (\pm 17)$. For subject 4, γ was much more variable (reason not yet identified). It was also noted that γ sometimes increased significantly towards the end of the 5-hour period of iontophoresis (Fig. 6).

Figure 5a-d



CONCLUSIONS

- o Lactate is easily extracted by iontophoresis. However, considerably more research is required to:
 - i. Link interstitial lactate (extracted by iontophoresis) with blood lactate.
 - ii. Eliminate artifacts (sweat, skin depot and consumer products).
 - iii. Understand the important inter and intra-variability observed.

REFERENCES

- (1) Le Boulanger et al., *Physiol.Meas* 25 (2004) R35-R50.
 - (2) Tamada et al., *JAMA* 282 (1999) 1839-1844.
 - (3) Le Boulanger et al., *Clin. Chem.* (2004) In Press.
- Published online June 10, 2004.

ACKNOWLEDGEMENTS

Funded by the USAMRAA (DAMD17-02-1-0712) and the US National Institutes of Health (EB 001420). The information presented does not necessarily reflect the position or the policy of the U.S. Government, and no official endorsement should be inferred.

APPENDIX 10

Simultaneous Extraction of Urea and Glucose by Reverse Iontophoresis *in Vivo*.



¹A.Sieg, ^{1,2}M.B. Delgado-Charro and ^{1,2}R.H. Guy

¹School of Pharmacy, University of Geneva, Switzerland & ²Dept. of Pharmacy and Pharmacology, University of Bath, UK.

BACKGROUND

- o The Glucowatch Biographer® relies on reverse iontophoresis to measure glucose levels in diabetics.
- o The extraction efficiency varies inter- and intra-individually; as a result, the Glucowatch must be calibrated against a conventional blood sample.

OBJECTIVE

To avoid the calibration step via the use of an "internal standard".

The specific aims of this work, are to investigate *in vivo*:

- o whether transdermal iontophoresis can be used to simultaneously extract urea and glucose.
- o the use of urea as an internal standard for glucose extraction, i.e., the validity of the following equation:

$$\frac{J_{\text{glucose}}}{J_{\text{urea}}} = K \frac{C_{\text{glucose}}}{C_{\text{urea}}}$$

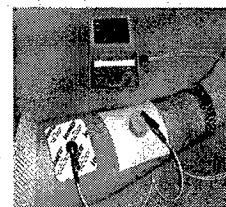


Figure 1

METHODS

Study population:

- o Nondiabetic subjects (25-40 years; 1 male, 5 females) with no history of skin disease. 5 subjects participated once, 1 subject participated twice. The protocol was approved by the University of Geneva IRB. Informed consent was obtained.

Iontophoresis protocol:

- o Direct current (0.6 mA \approx 0.3 mA/cm²; Ag/AgCl electrodes) was passed for a total of 5 h.
- o The cathodal solution was 1.2 mL of a pH 8.5 10mM Tris-buffer. The anodal solution was 100 mM NaCl in the same buffer.
- o Every 15 min post-initiation of the current, the entire cathodal solution was collected and replaced by fresh buffer.
- o After 2.5 h of iontophoresis the subjects ingested an oral glucose solution (75 g/300 mL water). From this point onward, glycemia was measured before each subsequent 15-min collection interval using a conventional glucose monitor (Glucotrend 2).
- o Blood urea levels were determined by three spot measurements using capillary blood (Reflotron Benchtop Analyser), immediately before starting iontophoresis, at the time of the first blood glucose measurement, and directly after current termination.

Sample analysis:

- o Glucose was assayed by high-performance ion chromatography with pulsed amperometric detection.
- o Urea was determined colorimetrically.

RESULTS and DISCUSSION

1. J_{glucose} tracked C_{glucose} faithfully when the volunteers were challenged with an oral glucose load (Figures 2.A-C).
2. J_{urea} remained quite stable reflecting the fact that C_{urea} did not change appreciably during the experiment (Figures 2.A-C).
3. The variability in the normalized extraction flux of urea ($J_{\text{urea}}/C_{\text{urea}}$) was on the order of 25%, that for glucose was greater (>45%) (Figure 3).
4. The ratio $J_{\text{glucose}}/J_{\text{urea}}$ divided by the ratio of the systemic concentrations ($C_{\text{glucose}}/C_{\text{urea}}$) yielded an extraction coefficient (K) that could be compared between subjects (Figure 4).
5. It was found that $K = 0.45 \pm 0.25$.

Figures 2.A-C

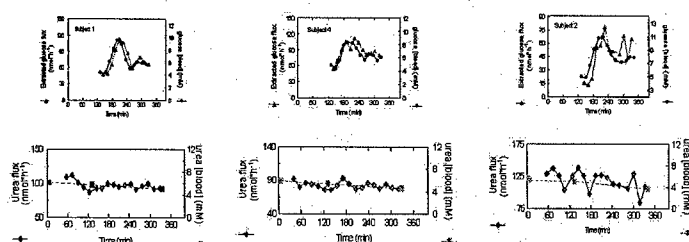


Figure 3

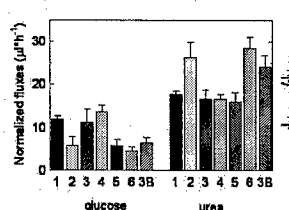
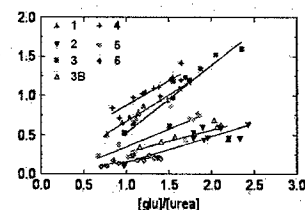


Figure 4



CONCLUSIONS

- o Urea performed reasonably as an internal standard. Both its extraction flux and systemic concentration remained quite constant.
- o Normalization of glucose fluxes with urea may correct for glucose extraction variability. However, additional work is necessary to determine the contribution of other factors which cause variability in glucose extraction.

ACKNOWLEDGEMENTS

Funded by the USAMRAA (DAMD17-02-1-0712) and the US National Institutes of Health (EB 001420). The information presented does not necessarily reflect the position or the policy of the U.S. Government, and no official endorsement should be inferred.